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(54) Title: THERAPEUTICAL COMPOSITION AGAINST PSORIASIS ON MEDICINAL HERB BASIS

(57) Abstract

The present invention relates to therapeutical compositions on medicinal herb basis for the treatment of psoriasis. Another object of the invention is the preparation of the said compositions. A further object of the invention is a cosmetic composition containing the same medicinal herbs. The therapeutical composition is characterized by comprising as active ingredient the organic solvent extract of the following medicinal herbs: 4 to 20 parts by weight of celandine, 4 to 20 parts by weight of St. John's wort, 0 to 4 parts by weight of achillea, 0 to 15 parts by weight of burweed, 0 to 8 parts by weight of marigold and 0 to 6 parts by weight of camomile.

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THERAPEUTICAL COMPOSITION AGAINST PSORIASIS ON MEDICINAL HERB BASIS

Technical field

The present invention relates to therapeutical compositions on medicinal herb basis for the treatment of psoriasis. Another object of the invention is the preparation of the said compositions. A further object of the invention is a cosmetic composition containing the same medicinal herbs.

Background art

Psoriasis constitutes one of the most common chronic skin diseases. It is a genetically determined, frequently hereditable skin disease, which appears mostly in the age of 20 to 30, and accompanies the patient through the whole life. Accordingly, only the symptoms thereof can be eliminated mostly for a certain period only, but curing is not possible yet.

Characteristic symptoms of the disease include enhanced hornification, erythema and infiltration. Internally, the disease may be associated with articular symptoms known as arthropathea psoriatica.

The pathogenesis includes two important features:

- epidermopoesis enhanced to multiple of the normal as well as the morphological and biochemical modifications associated therewith. Epidermopoesis is 7 to 8-fold of the normal value. The "turn over" period of the intact epithelium is 27 days wile the same is 3 to 4 days in case of psoriasis;

- the pathologically enhanced epidermopoesis can be induced with impulses on clinically intact skin of psoriatic patients (Köbner-sign).

A large number of compositions are used for external treatment of psoriasis. These are used in case of mild and medium grave indications. The grave and complicated cases need internal treatment as well. For internal treatment especially cytostatica (e.g. Methothrexat) and aromatic retinoids (Tigazon) are used. Both compositions are of strong effect, which may, however, associated with serious, sometimes even perilous side effects. Accordingly, the administration may be effected under strict medical control only.

In the external therapy, salicyl is widely used in a concentration of 5 to 10 %, in vaseline base. Salicyl dissolves squamae rapidly, but has a minimal influence on erythema infiltrations. Accordingly, the combination thereof

with steroids proved to be more effective. These combination contain generally 3 to 5 % of salicyl and usually fluorinated steroid. These compositions are mostly magistral but also commercial products are available, e.g. Loriden A. Preferred salicyl-steroid combination against psoriasis arethe Diprosalis paint and ointment compositions. These are of very strong effect; the salicyl dissolves squamae while the steroid component suppresses the erythema and infiltration. However, strong steroids when using on a large body surface and for a long period tend to be absorbed (which is especially characteristic in case of psoriasis) and might cause adrenocortical depression. Steroids also predispose to bacterial and fungal superinfection and may cause dermatrophia and haemorrhage. The effect thereof is only temporary and in most of the cases a ready recidiva takes place.

Tar ointments have the disadvantage that only a part of the body surface is recommended to be treated as absorption from larger surface might damage the kidneys. Also patients do not like these compositions as they contaminate clothes. A combination with steroids is Loriden Tar. Tar may cause serious inflammations, and use on hairy skin is not advisable due to the danger of folliculitis.

The composition Psoriazin could not fulfil the requirements. This ointment contains mustard gas, in a dilution of 1 : 20,000. Mustard gas is strongly irritative and only a few patients can tolerate.

Ditranol has been used in the treatment of psoriasis for long. In a more dilute form (¼ - ½ %) the ointment caused a slow, continuous improvement, however, nowadays the so-called "flush" treatment with a more concentrated composition is more preferred, wherein the daily treatment takes ½ to 1 hour followed by careful removal of the rests. The composition is highly effective, but very drastic; it induces serious inflammations and accordingly, treatment may be performed only under clinical conditions, home treatment should be avoided.

PUVA and SUVA treatment is also used in case of psoriasis. The apparatus emits selective PUVA, the so-called "black light". In the PUVA treatment photosensibilisator is also added, or possibly 6-methoxy-psoralen is applied by painting. The apparatus is, however, very difficult to obtain and very expensive. The scope of contraindications is very broad and accordingly, the number of possible treatments is limited.

The Psoricur oil is based on the oily extract of different medicinal herbs, among others Allium sativum (garlic). This composition is described in the specification PCT/HU87/00060 published under WO89/05651. Recent experiments showed that the treatment induced frequently serious inflammations. On the other hand, the treatment is quite expensive.

In our Hungarian Patent No. 196,557 a composition comprising organic solvent extract of medicinal herbs in natural carrier is described. The composition is a brownish-

green ointment which is easy to smear and is well-penetrating. Application is suitably made in the evening by rubbing in the skin several times. The composition possesses sufficient therapeutical activity, within a few days squama formation ceases and erythema and infiltration decreases, first in the center of the plaque, while the peripherial ring disappears at last. Symptom-free state was observed after 5 to 6 months, in some cases 2 to 3 months. As the said composition consists of natural materials only, use on even the entire body surface is also possible, and home treatment is also possible considering that no specific external side-effects were observed, like inflammation, folliculitis, fungal , bacterial complications, etc.

However, also this composition possesses some drawbacks, e.g. the effect is too slow, and the use thereof may be hazardous to health as one of the components, i.e. birthwort (Aristolochia folium) is toxic and regarded as carcinogenic, and accordingly, use thereof is also prohibited in different countries.

Accordingly, the present invention aims to provide a composition which serves for a permanent suppression of psoriasis, possesses no toxicity, can be used in the home practice without limitations, even more times a day and the effect appears within a short treatment period, the effect is prolonged and no symptoms reappear on the recovered skin surface.

Disclosure of the invention

The present invention provides a composition against psoriasis which comprises the extract of a combination of the medicinal herbs celandine and St.John's wort as well as optionally achillea, burweed, marigold and camomile as active ingredient. The composition preferably contains at least one dermatologically acceptable pharmaceutical or cosmetical additive.

The book Rápóti, J., Romvári, V.: Medicinal plants (1975) provides the following information about the above plants:

1. Burweed:

The drug (Bardanae radix) contains a small amount of volatile oils, tannic material, resin, sterins, 20 to 50 % of inulin, mucin, fats, sugar, organic acids as well as bitter materials. The decoction per se or tea blend is used as diuretic, biliary and renal calculus litholytic, blood purifying, choleretic agent. Externally, the decoction is used against herpes, eczema, impetigo, as rinsing, against pharyngitis as gargle. As hair care cosmetical agent may also be used.

2. Celandine:

The drug (Chelodinii herba) and root (Cheladonii radix) have a bitter, irritating taste, the powder thereof, when inhaling, stimulates coughing and sneezing. The drug contains several alkaloids, resins, volatile oils, vitamin C, nicotinic acid, etc. The active material content of the root is richer than that of the drug. Both drugs are used for preparing medicines against bilious and liver disorders in large scale. The drugs per se cannot be used for preparing tea; also the use therof in tea blends should be confirmed by physician. The juice of fresh plant is used to eliminate corn and wart.

3. Camomile:

The drug (Chamomile flos) contains 0.2 to 0.7 % of dark blue volatile oil with a sulene content of 3 to 5%, as well as glucosides, tannic material, bitter components, vegetable acids and sugar.

The camomile flower is one of the most commonly known and used domestic medicines, also registered in the pharmacopeiae of most countries. The decoction thereof possesses the following effects: tranquillizing, spasmolytic, carminative, stomachic, digestive, diaphoretic, disinfectant, gastrointestinal disease curing effects. Externally, it is used

as eye pack, throat gargle and gingival recuperative agent. It can be used not only as medicinal tea but also as regular drink due to the pleasant taste thereof.

4. Marigold:

The drug (Calendula flos sine calicibus) contains carotene, glucosides, bitter components, small amount of volatile oil and color materials. The decoction is used against gastric and intestinal ulcer externally, as well as for packing slowly healing wounds and ulcer. The colouring component thereof is used for colouring food, medicines an drinks.

5. St.John's wort:

The drug (Hyperici herba) contains glucosides, tannic material, colouring agents, sugar, choline, pectin, nicotinic acid, etc. The tea is used against gastric and intestinal ulcer, hypertension, digestive troubles, internal and external bleedings, renal and bilious diseases. Externally, the decoction is used in the treatment of slight skin disorders and slowly healing wounds. As rinsing agent, it is recommended against pharyngitis and paradentosis.

6. Achillea:

The drug (Millefolii herba) contains an azulene-containing, blue volatile oil in an amount of 0.1 to 1.5% as well as the glucoalkaloid achillein, bitter components, tannic material, conitic acid, aspargene, glycoside, fatty oil, resin, carbohydrate. The achillea oil is the active component of anti-inflammatory ointments, e.g. for the treatment of eyes, as well as in facial creams and skin-care compositions. The azulene ointment does not suppress inflammation, but accelerates the process. The drugs and teas of achille are used as appetizers, digestives, spasmolitica, for promoting bile- and liver functions, eliminating menstrual disorders, curing urinary and respiratory disorders and hypertension. The indication also includes the treatment of varicosity, enteritis and gastroenteritis, as well as prostatic disorders. Externally, the decoction serves as gargle and pack against gingival, eye, and general inflammations.

The active material of the composition according to the present invention is the extract of the following components:

celandine	4 to 16 parts by weight
St. John's wort	4 to 20 parts by weight
achillea	0 to 4 parts by weight
burweed	0 to 15 parts by weight
marigold	0 to 8 parts by weight
camomile	0 to 6 parts by weight.

Especially preferred are the compositions of the present invention which contain achillea as a compulsory component.

The medicinal herb extract used as the active component of the composition of the present invention is obtained by organic solvent extraction. As organic solvent, preferably a polar solvent, e.g. ethyl acetate, an alcohol or aceton is used. Especially preferred is the use of aceton.

The carrier of the composition may be any pharmaceutical or cosmetic carrier which can be used on the skin. The compositions are suitably in the form of cream, ointment or oily elixir. As carrier for the preparation of the present compositions suitably linseed oil, cholesterol, vaseline, Adeps lanae and distilled water can be used. Preferred carriers are the linseed oil and cholesterol.

The ratio of the active material - i.e. the medicinal herb extract - and the carrier is not a critical factor and may vary between broad ranges, It is important, however, that the carrier must be present in an amount sufficient to retain the composition on the surface, and to ensure penetration through the skin.

The compositions according to the invention can be prepared e.g. by milling the above dry herbs, followed by extracting suitably each separately in a suitable amount of polar organic solvent at a temperature not exceeding 60°C, filtering the mixtures, combining the filtrates, followed by

evaporating to dry. After evaporation, the active ingredients are dissolved in a solvent which is not skin irritative, e.g. ethanol, and incorporated as a solution in the suitable carrier(s). Under continuous stirring, the composition is filled into tubes, jars and flasks, resp., sealed and stored in dry, cool conditions.

According to a preferred embodiment of the invention, the mixture of cholestrol, Vaselinum album, Cera lanae, Oleum lini and Aqua destillatum are used to formulate a cream. A composition for use on psoriasis of hairy scalp can be obtained preferably by using Oleum linii containing suitably 0.01 % of α-d,l-tocopherol, i.e. vitamin E, as natural antioxidant. The oil composition is filled into flasks or flacons.

Application of the compositions is accomplished by smearing the skin surface several times daily or only once but for a longer period, e.g. 2 to 3 hours. Treatment of psoriatic skin surface results in ceasing of squama formation within one week. During this period the characteristic tensive, burning and itching feeling disappears or substantially decreases. Subsequently, also erythema and infiltration tend to disappear, first in the plaque center and finally, only a peripherial ring remains.

The continuous pachydermia, the most painful form of which appears on palm and sole, needs a longer treatment, i.e., even by a daily treatment of 2 to 3 hours recovery

needs a longer period. However, even in these cases a rapid and substantial recovery will be observed, pain is strongly subsided, tension, haemorrhage and clefts slowly disappear. In the first period, only the number thereof decreases rapidly, then the pathologically thick skin begins to desquamate, erythema is formed on large area in place of the squamae, followd by intensive disappearance thereof, and thus, a normal epidermis is gradually forming. The limbs, in contrary to the previous state, can be used properly. The regeneration of skin of youngers and those having browner skin needs generally less time.

In certain cases, following a longer treatment period, reappearance of the characteristic symptoms was observed, however, a short treatment resulted in a very quick recovery.

Surprisingly it has been found that the compositions of the present invention can be used not only in the tratment of psoriasis but also on other pathological dermatitis. Thus, rubors, desquamation and induration of the skin can also be prevented by using the compositions. Esthethical appearance of the skin having been treated and regularily cared improved substantially. Accordingly, the present compositions may be used also as cosmetics.

The compositions of the present invention have the advantage that they consist of natural ingredients exclusively, which are unharmful to the human organism, possessing at the same time desinfecting and skin nutritioning effect. This

effect meets recent tendencies which prefers natural pharmaceutical products and cosmetics to synthetic compositions. Their use is very simple and can be applied on the same surface several times daily.

A most preferred application form is the use for treating psoriasis of hairy scalp in the form of an oily composition, which treatment was very difficult in the past.

The following examples serve for illustrating the present invention more in details:

Best mode of carrying out the invention

Example 1

Extract of medicinal herbs was prepared from the following dry plants:

burweed	120 g
celandine	100 g
St.John's wort	100 g
marigold	60 g
camomile	50 g
achillea	40 g

Each of the above plants were separately milled into powder, extracted in a suitably apparatus with acetone at a temperature not exceeding 60°C, the extracts were filtered, combined and evaporated at a temperature not exceeding 60°C,

followed by cooling to room temperature. The extract thus obtained was dissolved in 96,3% ethanol at about 50°C and used in the preparation of 5,000 g pharmaceutical composition.

Example 2

The medicinal herb extract obtained in Example 1 was formulated into pharmaceutical composition as follows:

250 g of Cholesterinum, 1,500 g of Vaselinum album, 1,500 g of Cera lanae, 1,600 g of oleum linii and the extract obtained in Example 1 (filled up to 150 g with Aqua dest.) were homogenized by stirring and filled into tubes under continuous stirring at 45 to 50°C. The brownish-green cream thus obtained was stored in cool place.

Example 3

For preparing a composition especially for treating psoriasis of hairy scalp the extract of Example 1 was diluted with Aqua dest. and stirred into 5000 g of Oleum linii containing 0.01 % vitamin E under continuous stirring, the composition was filled into suitable flasks.

Example 4

Treatments were carried out with the composition of Example 1 on a total of 92 patients. Treatment was performed once a day in the evening. After showering, the patients were treated by rubbing the composition in the skin, while one hour later the non-absorbed ointment rests were removed. The control group consisted of 60 patients, treated with 5% salicylic acid or fluorinated steroid. The treatments were carried out in a period of 3 months.

The results of therapy are summarized as herebelow:

Symptom-free:	35%
Substantial improvement:	40%
Moderate improvement:	11%
No change:	13%
Worsening:	1%

Summarized, the effect of the composition was much better than that of the control group. No side effects, except for the one case, were observed.

The photosensibilizing and skin irritating effect of the composition of example 2 was tested on albino guinea pigs. During 3 months treatment negative results were obtained in both effects.

Claims

1. Therapeutical composition against psoriasis characterized by comprising as active ingredient the organic solvent extract of the following medicinal herbs: 4 to 20 parts by weight of celandine, 4 to 20 parts by weight of St.John's wort, 0 to 4 parts by weight of achillea, 0 to 15 parts by weight of burweed, 0 to 8 parts by weight of marigold and 0 to 6 parts by weight of camomile.

2. Cosmetic composition against psoriasis characterized by comprising as active ingredient the organic solvent extract of the following medicinal herbs: 4 to 20 parts by weight of celandine, 4 to 20 parts by weight of St.John's wort, 0 to 4 parts by weight of achillea, 0 to 15 parts by weight of burweed, 0 to 8 parts by weight of marigold and 0 to 6 parts by weight of camomile.

3. The composition according to claim 1 characterized in that the extract is a polar organic solvent extract.

4. The composition according to claim 1 characterized in that the extract is an acetone extract.

5. The composition according to claim 1 characterized by comprising at least one pharmaceutically acceptable carrier.

6. The composition according to claim 5 characterized in that the carrier is linseed oil.

7. The composition according to claim 5 characterized in that the carrier is cholesterol, vaseline, Adeps lanae, linseed oil and distilled water.

8. The composition according to claim 1 characterized in that 1,25 kg composition contains the extract of 30 g burweed, 25 g celandine, 25 g St.John's wort, 15 g marigold, 12,5 g camomile and 10,9 g achillea.

9. The composition of claim 8 characterized in that as carrier, one kg composition contains 62,5 g cholesterol, 37,5 g vaseline, 375 g Adeps lanae, 400 g linseed oil and 37,5 g distilled water.

10. Process for preparing therapeutical composition against psoriasis characterized in that 4 to 20 parts by weight of celandine, 4 to 20 parts by weight of St.John's wort, 0 to 4 parts by weight of achillea, 0 to 15 parts by weight of burweed, 0 to 8 parts by weight of marigold and 0 to 6 parts by weight of camomile are extracted with an organic solvent, and the extract thus obtained is formulated with at least one pharmaceutically acceptable carrier.

INTERNATIONAL SEARCH REPORT

International Application No PCT/HU 91/00014

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5]: A 61 K 35/78

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
Int. Cl.[5]:	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	FR, A1, 2 342 071 (LABORATOIRE DE THERAPEUTIQUES PHYSIOLOGIQUES) 23 September 1977 (23.09.77), see claims.	(1)
Y	WO, A1, 89/05 651 (PSORICUR LTD.) 29 June 1989 (29.06.89), see claims 1,2,6,7,10.	(1,5,10)

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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 May 1991 (08.05.91)	04 June 1991 (04.06.91)
International Searching Authority	**Signature of Authorized Officer**
AUSTRIAN PATENT OFFICE)	

Form PCT/ISA/210 (second sheet) (January 1985)

Anhang zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

Annex to the International Search Report on International Patent Application No. PCT/HU 91/00014

Annexe au rapport de recherche internationale relatif à la demande de brevet international n°.

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(58) Field of Search
UK CL (Edition O) A5B BE
INT CL[6] A61K 35/78
ONLINE: CAPLUS, NAPRALERT, WPI, CLAIMS, JAPIO

(54) Pharmaceutical compositions containing extracts of Tagetes (Marigolds)

(57) Pharmaceutical base preparations comprise extracts of Asteraceae Tagetes, erecta (African Marigold), patula (French Marigold) or signata (Dwarf Marigold) or minuta. Preferably the compositions also contain extracts of any or a combination of the following: Bennis perennis, Hypericum perforatum, Symphytum officinalis, Thuja occidentalis, Thuja plicata, Ruta graveolens, Rosmarinus officinalis or Calendula arvensis. The preparations are particularly intended for the treatment of fungal infections of the nails, skin lesions, or disorders of the bones or joints.

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A PHARMACEUTICAL PREPARATION

This invention relates to a pharmaceutical base preparation, and to pharmaceutical preparations containing the base preparation. The pharmaceutical preparations are useful in the treatment of lesions of the skin, bones, joints and nails including, for example, hyperkeratosis, dry eczema, psoriasis, inflammatory conditions and viral and fungal infections.

There are numerous topical medicaments and treatments available to practitioners for the treatment of lesions of the skin, bones, joints and nails. There are disadvantages in that some medicaments and treatments are not painless, are invasive and may be palliative or short-term in effect. There may also be painful complications. In addition, some treatments are inappropriate for patients with certain systemic diseases and for patients 'at risk'.

It is an aim of the present invention to provide pharmaceutical preparations useful in the treatment of lesions of the skin, bones, joints and nails, which pharmaceutical preparations are painless, non-invasive and effective in the longer term.

In accordance with a first aspect of the present invention, there is provided a pharmaceutical base preparation comprising flavonoids or glycosides of Asteraceae, the Asteraceae being Tagetes erecta orange, yellow or lemon-yellow, Tagetes minuta, Tagetes patula and Tagetes signata.

Preferably, the pharmaceutical base preparation is one in which the proportion of each individual component is 20%.

Preferably, the pharmaceutical base preparation is one in which the ingredients are fresh whole plant material. Preferably, the fresh plant material will be minced fresh plant material.

The pharmaceutical base preparation may include as a solvent 85% strength isopropyl alcohol. The pharmaceutical base preparation may be one in which the proportion of isopropyl alcohol is 20%.

In accordance with a second aspect of the present invention there is provided a pharmaceutical preparation for use in the treatment of skin lesions, which pharmaceutical preparation comprises the pharmaceutical base preparation, caretenoids of Compositae and Hypericeae, and terpenoids of Cupressaceae.

In the second aspect of the invention, the pharmaceutical preparation may be one in which the Compositae is Bellis perennis flowerhead or leaf, in which the Hypericeae is Hypericum perforatum flowerhead or leaf, and in which the Cupressaceae is Thuja occidentalis leaf.

The pharmaceutical preparation may include at least one of isopropyl alcohol, soft white paraffin, arachis oil, aqueous cream and aqueous gel.

Preferably, the pharmaceutical preparation is one in which the ingredients are in the form of an extract of minced fresh or powdered dried plant material.

In accordance with a third aspect of the invention, there is provided a pharmaceutical preparation for use in the treatment of bone and joint disorders, which pharmaceutical preparation comprises the pharmaceutical base preparation, alkaloids of Boraginaceae and Rutaceae, and terpenoids of Labiatae.

In the third aspect of the invention, the pharmaceutical preparation may be one in which the Boraginaceae is Symphytum officinalis flowerhead or leaf, in which the Rutaceae is Ruta graveolens flowerhead or leaf, and in which the Labiatae is Rosemary officinalis leaf.

The pharmaceutical preparation may include at least one of isopropyl alcohol, arachis oil and aqueous cream.

Preferably, the pharmaceutical preparation is one in which the ingredients are in the form of an extract of minced fresh or powdered dried plant material.

In accordance with a fourth aspect of the present invention, there is provided a pharmacentical preparation for use in the treatment of fungal infections of nails, which pharmaceutical preparation comprises the pharmaceutical base preparation, terpenoids of Cupressaceae, and terpenoids or carotenoids of Compositae.

In the fourth aspect of the invention, the pharmaceutical preparation may be one in which the Cupressaceae are Thuja occidentalis leaf and Thuja plicata leaf, and in which the Compositae is Calendula arvensis flowerhead or leaf.

The pharmaceutical preparation may include at least one of isopropyl alcohol, arachis oil and aqueous cream.

Preferably, the pharmaceutical preparation is one in which the ingredients are in the form of an extract of minced fresh or powdered dried plant material.

The pharmaceutical preparations may have a keralytic, surface analgesic, anti-psoriatic, anti-eczematic, anti-viral, anti-inflammatory and anti-fungal effect and may be used to treat hyperkeratotic lesions, dry eczema, psoriasis, inflammation of the skin, bones and joints, viral infections of the skin and fungal infections of the nails.

The pharmaceutical preparations may be such that the percentage weight or volume of each component in a preparation is from 3% to 10% depending on the form of the preparation.

The pharmaceutical preparations may include:

a) 3% of extract of minced fresh or powdered dried Tagetes erecta orange, yellow or lemon-yellow whole plant;

(b) 3% of extract of minced fresh or powdered dried Tagetes minuta whole plant;

(c) 3% of extract of minced fresh or powdered dried Tagetes patula whole plant;

(d) 3% of extract of minced fresh or powdered dried Tagetes signata whole plant;

(e) 3% to 5% of extract of minced fresh or powdered dried Bellis perennis flowerhead or leaf;

(f) 3% to 5% of extract of minced fresh or powdered dried Hypericum perforatum flowerhead or leaf;

(g) 3% to 5% of extract of minced fresh or powdered dried Symphytum officinalis flowerhead or leaf;

(h) 3% to 6% of extract of minced fresh or powdered dried Thuja occidentalis leaf;

(i) 5% to 10% of extract of minced fresh or powdered dried Ruta graveolens flowerhead or leaf;

(j) 5% of extract of minced fresh or powdered dried Rosmarinus officinalis leaf;

(k) 3% to 5% of extract of minced fresh or powdered dried Calendula arvensis flowerhead or leaf;

(l) 6% of extract of minced fresh or powdered dried Thuja plicata leaf;

(m) 73% to 76% isopropyl alcohol or arachis oil or soft white paraffin or aqueous cream or aqueous gel.

The pharmaceutical preparations may be such that they include as said flavonoids or glycosides or terpenoids or alkaloids or carotenoids one or more of kaempferol, quercetagetrin, thiophene, tagetiin, symphytine, carotene, bellisoponin, thujone, thujaplicin, hypericine, borneol and graveoline.

Preferably, the pharmaceutical preparations are in the form of a paste, tincture, oil, ointment, cream or gel. The pharmaceutical preparations may be prepared as a paste with the pharmaceutical base preparation, a tincture with isopropyl alcohol, an oil with arachis oil, an ointment with soft white paraffin, a cream with aqueous cream, or a gel with aqueous gel.

The pharmaceutical preparations of the present invention have the important advantage over known compositions that they are non-invasive, painless and effective in the longer term in resolution of hyperkeratotic lesions, inflammatory conditions of the skin, bones and joints, in relief of pain, in the elimination of viral and fungal infections and in the resolution of psoriatic and eczematic lesions of the skin.

The composition of the invention comprises preferably the flavonoids or glycosides or terpenoids or carotenoids or alkaloids contained in the preferred plants set out in the following table.

Table

Plant family	Latin name of preferred plant	Name of chemical compound
Asteraceae	Tagetes erecta	kaempferol
	Tagetes minuta	quercetagetrin
	Tagetes patula	thiophene
	Tagetes signata	tagetiin
Boraginaceae	Symphytum officinalis	symphytine
Compositae	Calendula arvensis	carotene
	Bellis perennis	bellisoponin
Cupressaceae	Thuja occidentalis	thujone
	Thuja plicata	thujaplicin
Hypericeae	Hypericum perforatum	hypericine
Labiatae	Rosemary officinalis	borneol
Rutaceae	Ruta graveolens	graveoline

The pharmaceutical preparations of the present invention may be used in allopathic or homoeopathic or herbal form as topical applications alone or in conjunction with podiatry, including a cavity pad made up of chiropody felt according to the lesion and its location.

The invention will now be described with reference to the following non-limiting Examples.

EXAMPLE 1

The following ingredients were mixed to prepare a pharmaceutical base preparation which is useful in the preparation of a pharmaceutical preparation for the treatment of lesions of the skin, bones and nails.

Minced fresh T.erecta whole plant (orange, yellow or lemon-yellow)	20%
Minced fresh T.minuta whole plant	20%
Minced fresh T.patula whole plant	20%
Minced fresh T.signata whole plant	20%
85% isopropyl alcohol	20%
	100%

EXAMPLE 2

The following ingredients were mixed to prepare a pharmaceutical paste preparation which is useful in the treatment of skin lesions.

Pharmaceutical base preparation according to Example 1	80%
Extract of Bellis perennis flowerhead or leaf	5%
Extract of Hypericum perforatum flowerhead or leaf	5%
Extract of Symphytum officinalis flowerhead or leaf	5%
Extract of Thuja occidentalis leaf	5%
	100%

The pharmaceutical paste preparation was prepared and used as a surface analgesic and a keralytic, anti-eczematic, anti-psoriatic and anti-viral agent in the treatment of hyperkeratotic lesion, dry eczema, psoriasis and viral infections of the skin.

EXAMPLE 3

The following ingredients were mixed to prepare a pharmaceutical tincture preparation which is useful in the treatment of skin lesions.

Extract of T.erecta orange/yellow/lemon-yellow whole plant	3%
Extract of T.minuta whole plant	3%
Extract of T.patula whole plant	3%
Extract of T.signata whole plant	3%
Extract of Bellis perennis flowerhead or leaf	3%
Extract of Hypericum perforatum flowerhead or leaf	3%
Extract of Symphytum officinalis flowerhead or leaf	3%
Extract of Thuja occidentalis leaf	3%
70% isopropyl alcohol	76%
	100%

The pharmaceutical tincture preparation was prepared and used as a surface analgesic and keralytic, anti-eczematic, anti-psoriatic and anti-viral agent in the treatment of skin lesions.

EXAMPLE 4

The ingredients in Example 3, with the exception of isopropyl alcohol which was replaced by arachis oil, were mixed to prepare a pharmaceutical oil preparation which is useful in the treatment of skin lesions and used as a post-treatment emollient.

EXAMPLE 5

The ingredients in Example 3, with the exception of isopropyl alcohol which was replaced by soft white paraffin, were mixed to prepare a pharmaceutical ointment preparation which is useful in the treatment of skin lesions.

EXAMPLE 6

The ingredients in Example 5, with the exception of isopropyl alcohol which was replaced by aqueous cream, were mixed to prepare a pharmaceutical cream preparation which is useful in the treatment of skin lesions.

EXAMPLE 7

The ingredients in Example 5, with the exception of isopropyl alcohol which was replaced by an aqueous gel, were mixed to prepare a pharmaceutical gel preparation which is useful in the treatment of skin lesions.

Case history 1: Skin Condition

A female patient aged 70 suffered for 20 years from painful corn and callosity (hyperkeratosis) in the plantar area of both feet. She was treated with a pharmaceutical paste preparation prepared according to Example 2, a pharmaceutical tincture preparation prepared according to Example 3, and a pharmaceutical oil preparation prepared according to Example 4 for skin conditions in conjunction with protective pad once a week for three weeks. The patient experienced relief of pain within 48 hours and the corn and callosity on both feet completely disappeared within three weeks.

Method of application

The pharmaceutical tincture preparation was first applied to the lesion then the overlying callosity was removed with a scalpel. A protective chiropody felt pad with cavity was placed over the lesion and the pharmaceutical paste preparation was placed in the cavity which was then covered with a piece of micropore followed by adhesive strapping. The dressing remained in place for a week when the treatment was repeated once weekly for three more weeks. As brief immersion in water reactivates the chemicals in the paste, thereby enhancing the therapeutic action, during the treatment period the patient was able to take a bath, but for a few moments only. At the end of the clinical treatment the patient received pharmaceutical oil preparation and pharmaceutical tincture preparation for follow-up home treatment. The tincture was applied first over the treated area following which a few drops of oil were applied by gentle massage during the first week three times a day, the second week twice a day, the third week once a day and, as required, continued three times a week as preventive treatment.

EXAMPLE 8

The following ingredients were mixed to prepare a pharmaceutical paste preparation useful in the treatment of disorders of the bones and joints.

Pharmaceutical base preparation according to Example 1	80%
Extract of Symphytum officinalis flowerhead or leaf	5%
Extract of Ruta graveolens flowerhead or leaf	10%
Extract of Rosmarinus officinalis leaf	5%
	100%

The pharmaceutical paste preparation was prepared and used as a surface analgesic and anti-inflammatory agent in the treatment of disorders of the bones and joints and in particular hallux abducto valgus, hallux rigidus, hallux flexus, gout or any painful, inflamed joint or post-operative complication of surgery.

EXAMPLE 9

The following ingredients were mixed to prepare a pharmaceutical tincture preparation useful in the treatment of disorders of the bones and joints.

Extract of T.erecta whole plant orange,yellow or lemon-yellow	3%
Extract of T.minuta whole plant	3%
Extract of T.patula whole plant	3%
Extract of T.signata whole plant	3%
Extract of Symphytum officinalis flowerhead or leaf	5%
Extract of Ruta graveolens flowerhead or leaf	5%
Extract of Rosmarinus officinalis leaf	5%
70% isopropyl alcohol	73%
	100%

The pharmaceutical tincture preparation was prepared and used as a surface analgesic and anti-inflammatory agent in the treatment of disorders of the bones and joints and in particular hallux abducto valgus, hallux rigidus, hallux flexus, gout or any painful inflamed joint or post-operative complications of surgery.

EXAMPLE 10

The ingredients in Example 9, with the exception of isopropyl alcohol which was replaced by arachis oil, were mixed to prepare a pharmaceutical oil preparation useful as a post-treatment emollient in the treatment of disorders of the bones and joints and in particular hallux abducto valgus, hallux rigidus, hallux flexus, gout or any inflamed joint or post-operative complications of surgery.

EXAMPLE 11

The ingredients in Example 9, with the exception of isopropyl alcohol which was replaced by aqueous cream, were mixed to prepare a pharmaceutical cream preparation which is useful in the treatment of disorders of the bones and joints and in particular hallux abducto valgus, hallux rigidus, hallux flexus, gout or any inflamed joint or post-operative complication of surgery.

Case history: Bone conditions

A male aged 30 suffered from painful hallux abducto valgus and its associated condition bunion. He was treated with a pharmaceutical paste preparation prepared according to Example 8, a pharmaceutical paste preparation prepared according to Example 9, and a pharmaceutical oil preparation prepared according to Example 10 in conjunction with protective chiropody pad once a week for four weeks. He experienced relief of pain within 24 hours and reduction of inflamed soft tissues within four weeks.

Method of application

The pharmaceutical tincture preparation was applied over the bunion and a chiropody cavity pad was placed over it. The cavity was filled with the pharmaceutical paste preparation, covered with a piece of micropore and then strapped with adhesive plaster. The treatment was repeated once a week for four weeks. At the end of four weeks the patient received the pharmaceutical tincture and oil preparations for follow-up home treatment. The pharmaceutical tincture preparation was applied first and then a few drops of oil were applied by gentle massage over the bunion joint, first week three times a day, second week twice a day, third week once a day and therefore as required as a preventive measure.

EXAMPLE 12

The following ingredients were mixed to prepare a pharmaceutical paste preparation useful in the treatment of fungal infections of the nails.

Pharmaceutical base preparation according to Example 1	80%
Extract Calendula arvensis flowerhead and leaf	5%
Extract of Thuja occidentalis leaf	5%
Extract of Thuja plicata leaf	10%
	100%

The pharmaceutical paste preparation was prepared and used as an anti-fungal agent in the treatment of fungal infections of the nails.

EXAMPLE 13

The following ingredients were mixed to prepare a pharmaceutical tincture preparation useful in the treatment of fungal infections of the nails.

Extract of T.erecta whole plant orange, yellow or lemon-yellow	3%
Extract of T.minuta whole plant	3%
Extract of T.patula whole plant	3%
Extract of T.signata whole plant	3%
Extract Calendula arvensis flowerhead or leaf	3%
Extract of Thuja occidentalis leaf	6%
Extract of Thuja plicata leaf	6%
70% isopropyl alcohol	73%
	100%

The pharmaceutical tincture preparation was prepared and used as an anti-fungal agent in the treatment of fungal infections of the nails.

EXAMPLE 14

The ingredients in Example 13, with the exception of isopropyl alcohol which was replaced by arachis oil, were mixed to prepare a pharmaceutical oil preparation useful in the treatment of fungal infections of the nails.

EXAMPLE 15

The ingredients in Example 13, with the exception of isopropyl alcohol which was replaced by aqueous cream, were mixed to prepare a pharmaceutical cream preparation useful in the treatment of fungal infection of the nails.

Case history 3: Fungal infection of nails

A male aged 22 suffered with onychomycosis of the fifth toenail. He was treated with a pharmaceutical tincture preparation prepared according to Example 13 and a pharmaceutical paste preparation for nails prepared according to Example 12 once a week for four weeks followed by home treatment using a pharmaceutical tincture preparation prepared according to Example 13 and a pharmaceutical oil preparation prepared according to Example 14 for four months. The fungus in the nail was completely eliminated and the nail grew normally within eight months from the first treatment.

Method of application

The pharmaceutical tincture preparation was applied to the nail which was reduced using a file. A chiropody cavity pad was placed around the nail and the cavity filled with the pharmaceutical paste preparation, covered with a piece of micropore and strapped with adhesive plaster. At the end of four weeks, the patient received the pharmaceutical tincture preparation and pharmaceutical oil preparation for follow-up home treatment. The pharmaceutical tincture preparation was applied first and then a few drops of pharmaceutical oil preparation by gentle massage, the first week three times a day, second week twice a day, third week once a day and thereafter until the condition was resolved.

CLAIMS

1. A pharmaceutical base preparation comprising flavonoids or glycosides of Asteraceae, the Asteraceae being Tagetes erecta orange, yellow or lemon-yellow, Tagetes minuta, Tagetes patula and Tagetes signata.

2. A pharmaceutical base preparation according to claim 1 in which the proportion of each individual component is 20%.

3. A pharmaceutical base preparation according to claim 1 or claim 2 in which the ingredients are fresh whole plant material.

4. A pharmaceutical base preparation according to any one of the preceding claims and which includes as a solvent 85% strength isopropyl alcohol.

5. A pharmaceutical base preparation according to any one of the preceding claims and in which the proportion of isopropyl alcohol is 20%.

6. A pharmaceutical preparation for use in the treatment of skin lesions, which pharmaceutical preparation comprises a pharmaceutical base preparation according to any one of the preceding claims, carotenoids of Compositae and Hypericeae, and terpenoids of Cupressaceae.

7. A pharmaceutical preparation according to claim 6 in which the Compositae is Bellis perennis flowerhead or leaf, in which the Hypericeae is Hypericum perforatum flowerhead or leaf, and in which the Cupressaceae is Thuja occidentalis leaf.

8. A pharmaceutical preparation according to claim 6 or claim 7 and including at least one of isopropyl alcohol, soft white paraffin, arachis oil, aqueous cream and aqueous gel.

9. A pharmaceutical preparation according to any one of claims 6 to 8 in which the ingredients are in the form of an extract of minced fresh or powdered dried plant material.

10. A pharmaceutical preparation for use in the treatment of bone and joint disorders, which pharmaceutical preparation comprises a pharmaceutical base preparation according to any one of claims 1 to 5, alkaloids of Boraginaceae and Rutaceae, and terpenoids of Labiatae.

11. A pharmaceutical preparation according to claim 10 in which the Boraginaceae is Symphytum officinalis flowerhead or leaf, in which the Rutaceae is Ruta graveolens flowerhead or leaf, and in which the Labiatae is Rosemary officinalis leaf.

12. A pharmaceutical preparation according to claim 10 or claim 11 and including at least one of isopropyl alcohol, arachis oil and aqueous cream.

13. A pharmaceutical preparation according to any one of claims 10 to 12 in which the ingredients are in the form of an extract of minced fresh or powdered dried plant material.

14. A pharmaceutical preparation for use in the treatment of fungal infections of nails, which pharmaceutical preparation comprises a pharmaceutical base preparation according to any one of claims 1 to 5, terpenoids of Cupressaceae, and terpenoids or carotenoids of Compositae.

15. A pharmaceutical preparation according to claim 14 in which the Cupressaceae are Thuja occidentalis leaf and Thuja plicata leaf, and in which the Compositae is Calendula arvensis flowerhead or leaf.

16. A pharmaceutical preparation according to claim 14 or claim 16 and including at least one of the ingredients isopropyl alcohol, arachis oil and aqueous cream.

17. A pharmaceutical preparation according to any one of claims 14 to 16 in which the ingredients are in the form of an extract of minced fresh or powdered dried plant material.

18. A pharmaceutical preparation according to any one of claims 6 to 17 and which includes:

a) 3% of extract of minced fresh or powdered dried Tagetes erecta orange, yellow or lemon-yellow whole plant;

b) 3% of extract of minced fresh or powdered dried Tagetes minuta whole plant;

c) 3% of extract of minced fresh or powdered dried Tagetes patula whole plant;

d) 3% extract of minced fresh or powdered dried Tagetes signata whole plant;

e) 3% to 5% of extract of minced fresh or powdered dried Bellis perennis flowerhead or leaf;

f) 3% to 5% of extract of minced fresh or powdered dried Hypericum perforatum flowerhead or leaf;

g) 3% to 5% of extract of minced fresh or powdered dried Symphytum officinalis flowerhead or leaf;

h) 3% to 6% of extract of minced fresh or powdered dried Thuja occidentalis leaf;

i) 5% to 10% of extract of minced fresh or powdered dried Ruta graveolens flowerhead or leaf;

j) 5% of extract of minced fresh or powdered dried Rosmarinus officinalis leaf;

k) 3% to 5% of extract of minced fresh or powdered dried Calendula arvensis flowerhead or leaf;

l) 6% of extract of minced fresh or powdered dried Thuja plicata leaf; and

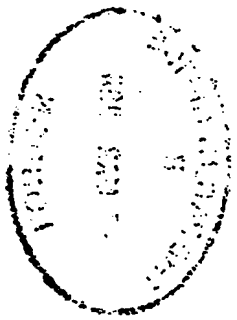
m) 73% to 76% isopropyl alcohol or arachis oil or soft white paraffin or aqueous cream or aqueous gel.

19. A pharmaceutical preparation according to any one of claims 5 to 17 and including as said flavonoids or glycosides or terpenoids or carotenoids or alkaloids one or more of kaempferol, quercetagetrin, thiophene, tagetiin, symphytine, carotene, bellisoponin, thujone, thujaplicin, hypericine, borneol and graveoline.

20. A pharmaceutical preparation according to any one of the preceding claims 5 to 18 and which is in the form of a paste, tincture, oil, ointment, cream or gel.

21. A pharmaceutical base preparation according to claim 1 and substantially as herein described.

22. A pharmaceutical preparation according to any one of claims 6 ,10 and 14 and substantially as herein described.



Amendments to the claims have been filed as follows

1. A pharmaceutical base preparation comprising Tagetes erecta orange, yellow or lemon-yellow, Tagetes minuta, Tagetes patula and Tagetes signata.

2. A pharmaceutical base preparation according to claim 1 in which the proportion of each individual component is 20%.

3. A pharmaceutical base preparation according to claim 1 or claim 2 in which the ingredients are fresh whole plant material.

4. A pharmaceutical base preparation according to any one of the preceding claims and which includes as a solvent 85% strength isopropyl alcohol.

5. A pharmaceutical base preparation according to any one of the preceding claims and in which the proportion of isopropyl alcohol is 20%.

6. A pharmaceutical preparation for use in the treatment of skin lesions, which pharmaceutical preparation comprises a pharmaceutical base preparation according to any one of the preceding claims, Bellis perennis flowerhead or leaf, Hypericum perforatum flowerhead or leaf and Thuja occidentalis leaf.

7. A pharmaceutical preparation according to claim 6 and including at least one of isopropyl alcohol, soft white paraffin, arachis oil, aqueous cream and aqueous gel.

8. A pharmaceutical preparation according to claim 6 or claim 7 in which the ingredients are in the form of an extract of minced fresh or powdered dried plant material.

9. A pharmaceutical preparation for use in the treatment of bone and joint disorders, which pharmaceutical preparation comprises a pharmaceutical base preparation according to any one of claims 1 to 5, Symphytum officinalis flowerhead or leaf, Ruta graveolens flowerhead or leaf and Rosmarinus officinalis leaf.

10. A pharmaceutical preparation according to claim 9 and including at least one of isopropyl alcohol, arachis oil and aqueous cream.

11. A pharmaceutical preparation according to claim 9 or claim 10 in which the ingredients are in the form of an extract of minced fresh or powdered dried plant material.

12. A pharmaceutical preparation for use in the treatment of fungal infection of nails, which pharmaceutical preparation comprises a pharmaceutical base preparation according to any one of claims 1 to 5, Thuja occidentalis leaf, Thuja plicata leaf and Calendula arvensis flowerhead or leaf.

13. A pharmaceutical preparation according to claim 12 and including at least one of the ingredients isopropyl alcohol, arachis oil and aqueous cream.

14. A pharmaceutical preparation according to claim 12 or claim 13 in which the ingredients are in the form of an extract of minced fresh or powdered dried plant material.

15. A pharmaceutical preparation according to any one of claims 6 to 14 and which includes:

a) 3% of extract of minced fresh or powdered dried Tagetes erecta orange, yellow or lemon-yellow whole plant;
b) 3% of extract of minced fresh or powdered dried Tagetes minuta whole plant;
c) 3% of extract of minced fresh or powdered dried Tagetes patula whole plant;
d) 3% of extract of minced fresh or powdered dried Tagetes signata whole plant;
e) 3% to 5% of extract of minced fresh or powdered dried Bellis perennis flowerhead or leaf;
f) 3% to 5% of extract of minced fresh or powdered dried Hypericum perforatum flowerhead or leaf;
g) 3% to 5% of extract of minced fresh or powdered dried Symphytum officinalis flowerhead or leaf;
h) 3% to 6% of extract of minced fresh or powdered dried Thuja occidentalis leaf;
i) 5% to 10% of extract of minced fresh or powdered dried Ruta graveolens flowerhead or leaf;
j) 5% of extract of minced fresh or powdered dried Rosmarinus officinalis leaf;
k) 3% to 5% of extract of minced fresh or powdered dried Calendula arvensis flowerhead or leaf;

l) 6% of extract of minced fresh or powdered dried Thuja plicata leaf; and

m) 73% to 76% isopropyl alcohol or arachis oil or soft white paraffin or aqueous cream or aqueous gel.

16. A pharmaceutical preparation according to any one of the preceding claims 5 to 15 and which is in the form of a paste, tincture, oil, ointment, cream or gel.

17. A pharmaceutical base preparation according to claim 1 and substantially as herein described.

18. A pharmaceutical preparation according to any one of claims 6, 9 and 12 and substantially as herein described.

The Patent Office

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Claims searched:	1-22	**Date of search:**	18 June 1997

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Other: ONLINE: CAPLUS, NAPRALERT, WPI, CLAIMS, JAPIO

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	GB 2083356 (KHAN M T) page 1 lines 12-22; Examples 1-4	1-5
X	US 4842859 (LIU Y) column 2 lines 17-20 & 33-35	1-5
X	WPI Abstract Acc. No. 97-050291 & RU2059698 C1 (CHERKASHINA N A) 1996 See abstract	1 at least
X	WPI Abstract Acc. No. 94-331252 & SU1819619 A1 (ZAPORO MED. INST.) 1993 See abstract	1 at least
X	WPI Abstract Acc. No. 89-215842 & JP010151504 A (SHIKOKU CHEM. IND. CO.) 1989 See abstract	1 at least
X	WPI Abstract Acc. No. 81-13163D & SU741880 B (ZAPORO MED. INST.) 1980 See abstract	1 at least

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Application No: GB 9604736.0 **Examiner:** Dr J Houlihan
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Category	Identity of document and relevant passage	Relevant to claims
X	Encyclopaedia of Common Natural Ingredients used in Food, Drugs and Cosmetics. 1980 "*Tagetes*" pages 302-303, especially "Folk Medicine".	1 at least
X	J. Pharm. Sci. Vol. 62 (6) 1973 Ickes G R *et. al.* "*Antitumor activity and preliminary phytochemical examination of Tagetes minuta (Compositae).*" pages 1009-1011	1 at least

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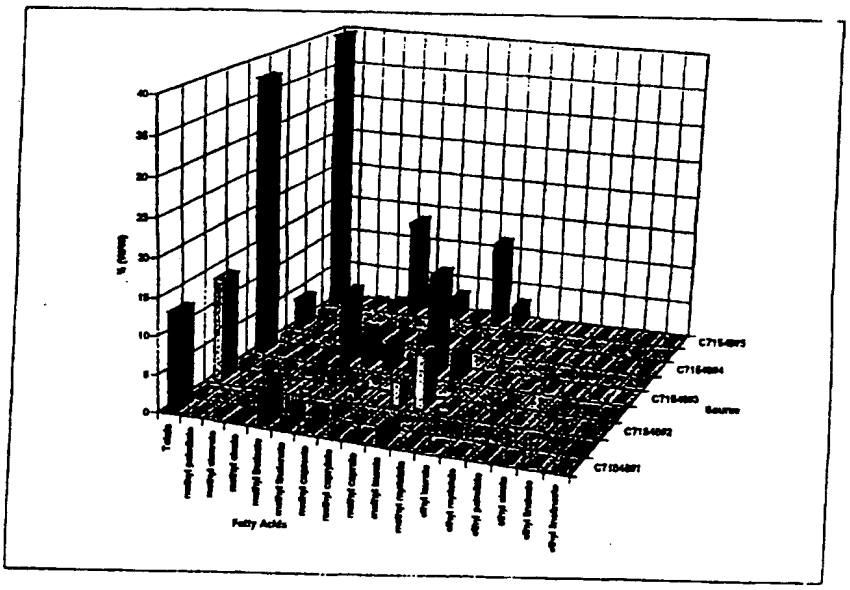

(57) Abstract

The present invention relates generally to botanical materials and methods for making such materials in medicinally useful and pharmaceutically acceptable forms. More particularly, the present invention relates to the use of compositional and activity fingerprints in the processing of botanical materials to produce drugs which qualify as pharmaceutical grade compositions which are suitable for use in clinical or veterinary settings to treat and/or ameliorate diseases, disorders or conditions.

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PHARMACEUTICAL GRADE BOTANICAL DRUGS

This is a continuation-in-part of co-pending U.S. Serial No. 08/632,273, filed on April 15, 1996, which is a continuation-in-part of U.S. Serial No. 08/421,993 filed on April 14, 1995 abandoned in favor of U.S. Serial No. 08/774,550, filed February 4, 1997.

1. FIELD OF THE INVENTION

The present invention relates generally to botanical materials and methods for transforming such materials into medicinally useful and pharmaceutically acceptable forms. More particularly, the present invention relates to the use of compositional and activity fingerprints in the processing of botanical materials to produce botanical drugs which qualify as pharmaceutical grade compositions which are suitable for use in clinical settings to treat and/or ameliorate diseases, disorders and/or conditions.

2. BACKGROUND OF THE INVENTION

Pharmaceutical manufacturing is based on control over the composition and bioactivity for each manufactured batch. This standardization and control provides reproducible material in the predictable and consistent treatment of patients. Herbal medicines, produced from botanical materials, have presented a unique problem for manufacturers desiring the control, reproducibility, and standardization that are required of pharmaceuticals. This problem is primarily due to the plurality of components contained in an herbal medicine and the large variation in composition and potency due to raw material growing and harvesting conditions.

Plants have been, and continue to be, the source of a wide variety of medicinal compounds. For centuries, various forms of botanically derived materials have been used to treat countless different ailments. The botanical materials have typically been in the form of powders made from of one

or more plants or plant parts or extracts derived from whole plants or selected plant parts. These powders and extracts are for the most part complex mixtures of both biologically active and biologically inactive compounds.

Although plant powders and extracts have been used widely for medicinal purposes, there are a number of problems associated with the use of such medicaments. For example, the complex chemical nature of the botanical materials makes it difficult to use the botanical materials in any type of controlled and predictable manner. The potential variations in the chemical composition of different batches of material obtained from different plant harvests makes such materials unsuitable for use in clinical situations.

On a positive note, the complex groupings of bioactive components typically found in botanical materials presents the potential for synergistic bioactivity profiles. However, these potential increases in medicinal effectiveness are not predictable due to the unknown nature of these complex materials.

The above problems associated with the inherent chemical complexity of botanical medicaments has resulted in a great deal of effort being directed to the separation and isolation of the biologically active components from numerous medicinally important botanical materials. This area of endeavor has expanded rapidly in conjunction with the many improvements in chemical separation and analysis technology. Once isolated and purified, the various active components are used in clinical settings to establish the medicinal effectiveness of a specific component. Separation and purification of individual components from botanical materials is the cornerstone of this type of drug development procedure. Once purified, the suspected active component is typically mixed with a pharmaceutically acceptable carrier and subjected to further studies in laboratory animals and eventual clinical trials in humans. Upon proof of clinical efficacy, these types of drugs are considered to be pharmaceutical grade because they contain a single or at most

a small number of well-characterized compounds which are present in known quantities.

Pharmaceutical grade drugs are advantageous in that they allow careful tracking of the effects of individual compounds in treatment protocols. Further, the dosage of the drug can be carefully controlled to provide relatively predictable medicinal action. A disadvantage of the relative purity of such pharmaceutical grade drugs is that the potential for complex and synergistic biological activity provided by naturally occurring plant materials is reduced because of the isolation of the drug from its natural environment. The study of isolated products may represent also artifacts produced by breakdown of sensitive biological/botanical complexes. The potential benefit provided by such synergistic activity is believed by many industry experts to be outweighed by the clinical risks associated with the use of complex plant materials which are not well characterized or controlled in a clinical setting.

Although isolation and purification of single compounds from plant materials has been a popular form of drug research and development, there also has been interest in studying complex botanical extracts to characterize their medicinal qualities. For example, as discussed below, mistletoe extracts have been studied in some detail.

Mistletoe belongs to the genus *Viscum* (family, *Loranthaceae*) which includes a variety of semiparasitic plants found all over the world. Mistletoe is a parasite which grows on a variety of deciduous trees including apple, cherry, oak, ash Hawthorn, lime and acorn. Mistletoe and extracts of mistletoe have been used for centuries in a wide variety of therapeutic settings. The effectiveness of mistletoe as a remedy for treating a multitude of ailments has been the subject of a great deal of folklore, superstition and mystical accounts. Although many of the early uses for mistletoe may have been based more on fantasy than on fact, the reputation of mistletoe as a powerful elixir is well deserved because this parasitic plant contains

a rather large variety of complex and pharmacologically potent components.

Beginning in the early 1900's, mistletoe and the pharmacological properties of extracts from mistletoe have been subjected to a more rigorous scientific investigation. In particular, mistletoe extracts have been suggested for use in treating a variety of specific diseases including cardiovascular illnesses, especially hypertension and arteriosclerosis; cancer and arthrosis. Fermented mistletoe extracts marketed under the tradenames ISCADOR®, HELIXOR®, and PLENOSOL® have been proposed for use in treating a number of specific diseases. ISCADOR® and HELIXOR® have been injected subcutaneously while PLENOSOL® has been administered both intracutaneously and intravenously. These three commercially available preparations are derived from mistletoe found in Europe, *Viscum album L.*

Since 1980, the investigation of mistletoe has increased due to its immunomodulatory properties and potential usefulness in treating HIV and cancer. See International Journal of Cancer Research Treatment-ONCOLOGY- Vol. 43, Supplement 1, 1986. A major problem facing mistletoe investigators has been the analysis, identification and standardization of the pharmacologically active components in mistletoe and extracts thereof. This problem is exacerbated by the fact that the numerous complex components which are found in mistletoe extracts vary widely in type and amount depending upon the species of mistletoe, the location where the plant is grown, the time of year when the plant is harvested, the particular host tree, the extraction procedure used and a number of other factors.

The principal classes of components in mistletoe which have been found to provide pharmacological activity include phenylpropanoids, lectins, phenylpropans, viscotoxins, alkaloids, flavonoids, lignans, amines, phenyl carboxylic acids and polysaccharides. Although the general classes of pharmacologically important compounds which are generally present in mistletoe have been identified, investigators have

not had a great deal of success with respect to standardizing the multitude of available extracts to establish if one or more components are responsible for the observed bioactivity and whether the specific components act together or may be effective individually. The extremely diverse nature of mistletoe extracts and the inherent variability in extract compositions makes it difficult to use the extracts to conduct clinical investigations.

The preceding discussion regarding mistletoe is exemplary of the state of the art with respect to the plant materials which have been studied in detail. Many other complex plant materials and extracts exist which have potent, but relatively unpredictable, medicinal properties. These materials are, for the most part, useless in a clinical setting because of the inherent risks involved with treating patients with poorly characterized materials which have no established batch consistency and which may differ widely in composition. Accordingly, there is a need to provide methods for standardizing such complex botanical materials so that they may be used more effectively in clinical research and patient treatments.

3. SUMMARY OF THE INVENTION

This invention provides a method for making a pharmaceutical grade botanical drug. The method is the process of "pharmaprinting." In one embodiment, the method comprises the steps of: providing a botanical material which comprises a plurality of components which have a given biological activity; removing a representative aliquot from the botanical material; separating the aliquot into a plurality of marker fractions wherein each of the marker fractions comprises at least one of the active components; determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the aliquot; and comparing the bioactivity fingerprint of the aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical

grade botanical drug to provide a bioactivity fingerprint comparison to determine whether the botanical material is a pharmaceutical grade botanical drug based on the bioactivity fingerprint comparison.

This invention also provides a method comprising the steps of: providing a botanical material which has a given biological activity, said botanical material comprising a plurality of components; separating a representative aliquot of the botanical material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active component; determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the representative aliquot; and comparing the bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade botanical drug to determine whether the botanical material is a pharmaceutical grade botanical drug.

In one embodiment, one or more of the marker fractions contain one active component.

The method may also comprise the additional steps of: determining the amount of the active components in each of the marker fractions to provide a quantitative compositional fingerprint of the aliquot and comparing both the quantitative compositional and bioactivity fingerprints with a quantitative compositional and bioactivity fingerprint standard to determine whether the botanical material is a pharmaceutical grade botanical drug. The method may also comprise the additional steps of: determining a total bioactivity of the aliquot of the botanical material and comparing the total bioactivity of the aliquot with that of a total bioactivity of a standard which has been established for a pharmaceutical grade drug.

The invention also provides a method for making a pharmaceutical grade botanical drug, the method comprising the steps of: providing a botanical material which comprises a plurality of components which have a given biological

activity and wherein each active component has a standardized bioactivity profile; removing a representative aliquot from the botanical material; separating the aliquot into a plurality of marker fractions wherein each of the marker fractions comprises at least one of the active components; measuring the amount of each of the active component(s) present in each of the marker fractions; calculating the bioactivity of each of the marker fractions based on the amount of each active component present and the standardized component bioactivity profile to provide a calculated bioactivity fingerprint of the aliquot; comparing the calculated bioactivity fingerprint of the aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade botanical drug to provide a bioactivity fingerprint comparison to determine whether the botanical material is a pharmaceutical grade botanical drug is obtained based on the bioactivity fingerprint comparison.

The method of the invention is useful to make a pharmaceutical grade botanical drug from any botanical material which has a given or desired biological activity. Preferably, the botanical material is an extract made from plant material such as an aqueous or organic extract such as an alcoholic extract or a supercritical carbon dioxide extract. Alternatively, the botanical material is a powdered plant material, a seed oil, an essential oil or the product of steam distillation. Preferably, the botanical material is a homogeneous material.

The botanical material may be any botanical material. Suitable botanical materials include, but are not limited to, materials of plants commonly known as Aloe, Bilberry, Black Cohosh, Chamomile, Chaste tree, Chestnut, Echinacea, Evening Primrose, Feverfew, Garlic, Ginger, Ginkgo, Ginseng (Asian), Goldenseal, Green tea, Guggulipid, Hawthorn, Ivy, Kava, Licorice, Milk Thistle, Passion Flower, Pumpkin, Pygeum, Rosemary, Siberian Ginseng, Saw Palmetto, St. John's Wort, Stinging Nettle, Valerian, etc. The botanical material may also be a mixture of plant materials including, but not

limited to, mixtures of materials of plants such as Saw Palmetto and Pumpkin seed; Echinacea and Goldenseal; St. John's Wort and Valerian.

In this invention the active component(s) may be one of the following chemicals: acetogenins, alkaloids, carbohydrates, carotenoids, cinnamic acid derivatives, fatty acids, fatty acid esters, flavonoids, glycosides, isoprenoids, macrocyclic antibiotics, nucleic acids, penicillins, peptides, phenolics, polyacetylenes, polyketides, polyphenols, polysaccharides, proteins, prostaglandins, steroids and terpenoids.

The bioactivity/clinical indication for the botanical may be associated with any disease, disorder or condition of humans or other animals. Thus the methods are useful to produce pharmaceutical grade botanical drugs for treatment and/or amelioration and/or prevention of human and/or veterinary diseases, disorders or conditions. Exemplary indications include, but are not limited to, an allergic/inflammatory disorder, a cardiovascular disorder, a cancer or a central nervous system disorder, a gastrointestinal disorder, a metabolic disorder, nausea or a disorder induced by a microbial organism or a virus.

In these methods, the aliquot may be separated into both biologically active and inactive components. Furthermore, the marker fractions may comprise a class of related components.

This invention also provides a method of preparing a pharmaprint for a pharmaceutical grade drug. Furthermore, this invention provides for a pharmaceutical grade drug prepared by the methods described above.

3.1. DEFINITIONS

The term "pharmaceutical grade" when used in this specification means that certain specified biologically active and/or inactive components in a botanical drug must be within certain specified absolute and/or relative concentration range and/or that the components must exhibit

certain activity levels as measured by a disease-, disorder- or condition-specific bioactivity assay. The disease, disorder or condition may afflict a human or an animal.

As used herein "components" means discrete compounds (*i.e.* chemicals) which either are present naturally in a botanical drug or have been added to the botanical drug so as to prepare a pharmaceutical grade botanical drug having components within a defined bioactivity range(s) and/or compositional range(s).

As used herein "active components(s)" means one or more component(s) for which the summation of the individual component(s) activity in a disease-specific bioassay accounts for a substantial portion of the observed biological activity of botanical material. Preferably, the summation of the active components' activities accounts of the majority or greater than 50% of the observed biological activity.

As used herein "fractions" typically means a group of components or class of structurally similar components having defined parameters such as solubility, molecular weight range, polarity range, adsorption coefficients, binding characteristics, chemical reactivity or selective soluability. Most frequently fractions will be the product of chromatographic separation techniques, *i.e.*, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin layer chromatography, affinity chromatography, size exclusion chromatography, liquid-liquid chromatography *e.g.*, counter-current chromatography or centripetal chromatography.

The present invention may be understood more fully by reference to the following: detailed description of the invention, examples of specific embodiments in Sections below and the appended figures.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of a procedure in accordance with the present invention which is used to establish standard chemical and/or bioactivity fingerprints against which subsequent processed botanical materials are compared during production of pharmaceutical grade drugs.

FIG. 2 is a schematic representation of a procedure in accordance with the present invention which is used to process botanical materials into pharmaceutical grade drugs.

FIG. 3 is a schematic representation of a procedure for isolating different classes of biologically active components.

FIG. 4 shows the result of the fractional analysis for Saw Palmetto from a commercially available product. The vertical axis is in weight/weight percent. The two axes are the fractions of the various fatty acids and fatty acid esters and the solvent system.

FIG. 5 shows the inhibition of specific ^{3}H-DHT binding by increasing concentrations of Saw Palmetto extract #3. The molar concentrations were calculated assuming a molecular weight of 200 for the active components.

FIG. 6 shows the inhibition of specific ^{3}H-DHT binding by increasing concentrations of lauric acid ethyl ester.

FIG. 7 shows the inhibition of specific ^{3}H-DHT binding by increasing concentrations of linoleic acid ethyl ester.

FIG. 8 shows the chemical analysis of five different commercially available Saw Palmetto products. The vertical axis is in weight/weight percent. The other axes are the fatty acids and fatty acid esters and the source material.

FIG. 9 shows the chromatographic steps for the analysis of the mistletoe extract.

FIG. 10 shows the fractional analysis for St. John's Wort. The vertical axis is in weight/weight percent. The horizontal axes are the fractions and the chemical constituents.

FIG. 11 shows the results of the chemical analysis for St. John's Wort. The vertical axis is the mg per 10 ml of product and the other axes are the chemical compounds and the commercial source.

FIG. 12 shows the fractional analysis for ginger. The vertical axis is in weight/weight percent. The remaining axes are fraction number and the specific chemical components.

FIG. 13 shows the results of the chemical analysis of five commercially available ginger products. The vertical axis is in mg per capsule. The horizontal axes are the ginger constituents and the commercial source.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1. METHODS OF PHARMAPRINTING

The present invention provides a method for producing botanical drugs which may be classified as being of pharmaceutical grade. The method is designated "Pharmaprinting." The pharmaceutical grade botanical drugs made by the method of the present invention are particularly well-suited for use in clinical studies and more importantly for use in treatment of patients. The method insures that the drug being used for a particular protocol will be of consistent quality and consistently suitable for use as human and veterinary prophylatic or therapeutic agents.

The present invention provides the ability to closely control the quality, dosing and clinical effectiveness of

botanical extracts and other botanical materials, *e.g.*, botanical extract and mammalian tissue derived biological preparation. One aspect of the present invention involves the establishment of the chemical and/or bioactivity fingerprint standards for various botanical materials. Once established, the fingerprint standards are used in drug production procedures to insure that the botanical materials meet pharmaceutical grade requirements. Specific quantitative and biological fingerprints are presented which have been established for a number of botanical materials as a further aspect of the invention. These fingerprints are useful for determining if a particular botanical material meets levels of pharmacological activity and composition requirements for a particular treatment regimen. Such a determination is important to insure that clinical studies and patient treatment with the botanical materials are based on consistent and verifiable extract composition parameters.

This invention is useful in providing botanical materials which are sufficiently characterized and whose compositions are consistent between batches, so that they can be precisely dosed and used effectively in clinical settings. The methods described herein provide an assurance that the results of a clinical trial will be reproducible.

Initially, a sample of the botanical material of interest is obtained. Many botanicals are commercially available as the raw material or as a processed extract. Often it is a botanical extract or other composition which is intended for use as a drug. The processed material may include a plurality of active components which exhibit a given biological activity and plurality of inactive components which do not directly exhibit the biological activity of interest. In one embodiment, an aliquot is removed from the botanical material and subjected to a quality assurance or standardization procedure. Preferably, the aliquot is a representative aliquot of a homogeneous botanical material. The procedure involves separating the aliquot of botanical material into a plurality of marker

fractions wherein each of the marker fractions includes at least one of the active components or in some cases one of the inactive components. The amount of active component or inactive component in each of the marker fractions is determined in order to provide a quantitative fingerprint of the aliquot. The degree of biological activity for each of the marker fractions is also determined to provide a biological activity fingerprint for the aliquot. The chemical and/or biological activity fingerprints of the aliquot are then compared to corresponding fingerprints which have been established for a pharmaceutical grade drug. If the fingerprints of the botanical match the standard fingerprints, then the botanical is identified as a pharmaceutical grade botanical drug. If not, then the botanical may be modified so as to provide a match with the standard fingerprints or may be rejected.

5.1.1. METHODS OF DEVELOPING A PHARMAPRINT

The method of developing a Pharmaprint for a botanical when a range of putative active components is known begins with a literature review. It involves reviewing the chemical literature, the biological literature, the published bioassays and clinical data for the botanical. Particularly useful sources of information are the NAPRALERT computer database managed by Dr. Norman Farnsworth in the Program for Collaborative Research in the Pharmaceutical Sciences, University of Illinois, Chicago; Leung and Foster, Encyclopedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics, 2nd Ed. John Wiley & Sons: New York, NY, 1996; Herbal Drugs and Phytopharmaceuticals, ed. N.G. Bisset, CRC Press:Boca Raton, FL, 1994; Duke, Handbook of Biologically Active Phytochemicals and Their Activities, CRC Press:Boca Raton, FL, 1992; Tyler and Foster "Herbs and Phytomedicinal Products" in Handbook of Nonprescription Drugs Berardi et al. eds., United Book Press, Inc.:Washington, DC, 1996. For a given indication, the literature must be studied to confirm that the putative active components are actually

associated with that disease state. In addition, if there are any bioassays known for the putative active components and known for the indication, the bioassays must be consistent with both the indication and the putative active components. The appropriate bioassay(s) is tied to a clinically relevant endpoint(s). The bioassay(s) should be quantitative over a wide concentration range. Typically, an IC_{50} curve (Inhibitory Concentration 50%) or an appropriate K_i curve is prepared. A thorough chemical and biological analysis of both putative active components and chromatographic fractions of the botanical is then performed. The results are analyzed to prepare a quantitative analysis of the biological activity for each of the chemical components in the sample. Then, the bioactivity of the sample as a whole is compared to the bioactivity of the individual components. At this point the individual chemical components can be correlated with a clinically relevant endpoint. Similar methodologies may be applied to bioassays measuring stimulatory effects.

Based on activity of the components individually and knowing the total activity, the components should, when combined, account for a substantial portion of the biological activity. Generally, the combined activity will account for at least 25% of the total activity.

Preferably, the summation of the individual active components' activities account for the majority or greater than 50% of the observed biological activity. More preferably, the isolated individual components are responsible for more than 70% of the activity. More preferable still, the isolated individual components are responsible for greater than 80% of the biological activity.

Another consideration will be to select as few active components as possible to be part of the Pharmaprint. Fewer active components are important for practical considerations in raw material acceptance and manufacturing. In this invention, a correlation is established between the relevant chemical components and the bioactivity. Once a satisfactory

correlation is established, it may not be necessary to perform the biological fingerprints on each sample. A chemical analysis of the appropriate components and/or marker fractions. Rather, each sample of the botanical of interest will suffice to account for most of the biological activity and establish that a given botanical sample is pharmaceutical grade.

In one embodiment, the present invention may involve one of the following procedures. One procedure, as schematically outlined in FIG. 1, involves establishing the compositional and bioactivity fingerprint standards for a given pharmaceutical grade botanical drug. Once the fingerprint standards are established, then the actual processing of the botanical into a pharmaceutical grade drug can be carried out as schematically outlined in FIG. 2.

The initial step in establishing the chemical and/or bioactivity fingerprint for a given botanical involves separating the extract or powder into one or more groups as represented by step 1 in FIG. 1. These groups are separated out and identified based on their potential as markers (which may or may not comprise active components) for the fingerprint which is to be established for the processed botanical material. The putative components or groups of putative components which are chosen and identified as potential markers will vary widely depending upon the botanical being processed and the pharmaceutical use. There should be at least two putative markers selected for each botanical. The number of potential markers is may be more than five and can be as high 15 to 20 or more for complex botanical extracts or powders. The potential markers are identified and selected, for the most part, based on their potential biological activity or contribution to biological activity for a given pharmaceutical application. For different indication the same botanical may be used for preparing an extract with a different extraction procedure in order to optimize the specific bioactive constituents. Markers which have no apparent biological activity by

themselves may be separated out and may be included as markers for use in the fingerprint. These "proxy" markers may be desirable as an internal standard where the markers' presence is indicative of other active components necessary to provide a substantial portion of the overall observed biological activity for the botanical drug.

The initial separation of the botanical into various groups of putative markers is accomplished by conventional separation techniques ranging from simple extraction to complex affinity chromatography including gel filtration chromatography, flash silica gel chromatography and reverse phase chromatography. Once the putative markers have been identified for a given botanical, then the bioactivity of each of the markers is determined as depicted by step 2 in FIG. 1. The particular bioassay used to determine bioactivity of the botanical is chosen based upon the intended use for the botanical. The bioassay preferably will provide a reflection of the putative markers' bioactivity with respect to the condition or indication which is to be treated with the botanical.

The bioassay results obtained in step 2 are used to identify the components having the desired bioactivity (step 3) and those which are less active or essentially inactive (step 4). Each of the groups identified in steps 3 and 4 is then analyzed quantitatively to determine the amount of each component present in each group. The results of the bioassays and quantitative compositional assays are then used to prepare a bioassay fingerprint and/or a chemical fingerprint for the botanical as depicted by step 5 in FIG. 1. As part of establishing the fingerprints for the botanical, acceptable ranges of bioactivity and/or chemical composition are determined. This is done primarily based upon establishing acceptable ranges of bioactivity and quantitative amounts for each marker which provide for the desired pharmacological activity of the processed material as a whole.

In addition, various combinations of active and inactive marker fractions may be evaluated to establish potential increases in desired bioactivity resulting from combinations of the active and inactive components.

The bioassay and quantitative fingerprints which are established in step 5 provide an accurate identification of the botanical which can be used in establishing the dosage regimens and treatment schedules which are necessary for clinical use. The dosage regimens and treatment schedules are established using conventional clinical methods which are commonly employed when investigating any new drug. The processed material which is used to determine the dosage and treatment schedules must be matched with and meet the requirements of the fingerprints established in step 5. This method insures that the dosage and treatment schedules are effective and reproducible since the processed materials used in the dosage and scheduling studies all have the same fingerprints in accordance with the present invention.

The bioassay and quantitative fingerprints which are determined by the general procedure as set forth in FIG. 1 are used as part of the manufacturing procedure for producing pharmaceutical grade botanical drugs. The fingerprints are used as part of a quality assurance or standardization procedure to insure that a given botanical contains the appropriate compounds and is processed correctly to provide a botanical drug which will perform the same clinically as the material which has been standardized and tested in accordance with the procedure set forth in FIG. 1.

An exemplary procedure for producing pharmaceutical grade botanicals in accordance with the present invention is shown schematically in FIG. 2. The botanical of interest 21 is first processed by extraction, powdering or other manufacturing process to form a processed botanical material 22. A sample of the processed material 22 is then analyzed to establish whether or not it matches the fingerprint requirements established during the standardization procedure of FIG. 1. This quality assurance or standardization

procedure is depicted at 23 in FIG. 2. If the processed material meets the previously established fingerprint requirements for the particular material, then it is approved as being of pharmaceutical grade as represented by step 24. If the material is close, but does not quite match the standard fingerprint, then it is modified as required to match the fingerprint standards (step 25). The modification of the processed material to meet fingerprint standards may be done by a variety of ways. The methods of further processing botanicals may including additional extraction of the botanical, selective extraction, selective processing, recombination of batches (*e.g.* mixing high and low dose batches to prepare the pharmaceutical grade material) or the addition of various compounds, as required. If the botanical is substantially outside the fingerprint ranges for both bioactivity markers and quantitative markers, then the batch is rejected (step 26).

In one embodiment, the quality assurance standardization step 23 used to determine if a given botanical is pharmaceutical grade involves obtaining a uniform sample, preferably a homogeneous sample, or aliquot of the botanical which is to be tested. The sample should include the active components which contribute to the observed biological activity of the material and produce the bioactivity and/or chemical fingerprint of the previously determined standard. The sample will also include one or more inactive components. Inactive components are those which may not have a direct measurable biological activity. Inactive components include the following categories: components with activity so low that they do not account for a substantial portion of the activity; components whose presence indicates the presence of other bioactive components and can act as proxy markers for these components; inactive components that are chemically or biologically inactive in the relevant assays. The sample is preferably only a small aliquot of the botanical material being tested. Accordingly, it is important that a uniform

sample, preferably a homogeneous sample, be obtained which is representative of the entire batch of material.

A more detailed schematic is shown in FIG. 3 showing the initial separation of the different components present in an aqueous extract of a botanical. Sequential extraction and precipitation are used to isolate the active components in either the aqueous or the organic phase. The scheme in FIG. 3 is particularly well suited for separating the classes of water-soluble active components from a botanical such as mistletoe.

An exemplary general method for separating plants into major classes of chemical components is set forth schematically in FIG. 3. Primarily fresh plants (including leaves, roots, flowers, berries and stem) should be used. Specific plant parts, such as the leaves, flowers, stems or root may be used if desired.

The specific part or whole plant may be frozen at liquid nitrogen temperature. This facilitates grinding and also preserves the integrity and potency of the active components.

The pulverized powder is extracted with distilled water repeatedly. If desired, the extraction may be carried out with hot water, alcohol, other organic solvents, aqueous alcohol, dilute acetic acid or any combination thereof. The actual temperature chosen is preferably close to or at the boiling temperature of water. It is preferred that the overall bioactivity of the aqueous extract be initially determined. The combined water extracts are subjected to a specific bioassay, *e.g.*, a test for inhibiting the growth of bacteria in Petri dishes if the drug is to be used as antibacterial. Alternatively, tests against cell cultures of cancer cells are conducted preferably if the drug is intended for use as an anticancer agent. From these data, bioactivity units contained in an extract per ml are calculated (bioactivity units are defined as the number of dilution of this extract needed to inhibit 50% growth of bacterium or cancer cell in test system). Similarly bioactivity units for

a stimulatory effect, *e.g.*, immunostimulation can be calculated.

For establishing fingerprints in accordance with the present invention, the plant is extracted according to the procedure as set forth in FIG. 3 to separate it into major components (*e.g.* saponins, terpenoids, lipids, alkaloids, nucleic acids, proteins and carbohydrates). Each separated group of components is tested for bioactivity as needed. This may point to activity (*e.g.* in protein and alkaloid factions as in *Viscum album*). The active class or classes of compounds are further separated into individual components by affinity chromatography, high pressure liquid chromatography, or gas chromatography. The components with major contribution towards biological activity are quantified on the basis of weight and specific bioactivity units. These components provide the fingerprint to establish the pharmaceutical requirements for the original herbal extract. The bioactivity units per ml of the pharmaceutical grade extract provide a way to establish exact doses for clinical studies.

Once the sample is separated into individual marker fractions, and at least one having at least one active component, each fraction is analyzed to determine the amount of active component therein and provide a quantitative fingerprint of the sample. The quantitation of each fraction can be achieved using any of the known quantitative analysis methods. Exemplary quantitation methods include gravimetric analysis, spectral analysis or the use of quantitative detectors, such as those used in gas chromatography or high performance liquid chromatography and other separation systems. Other suitable quantitative analytical methods include analysis by enzymatic, radiometric, colorimetric, spectrophotometric, fluorescent or phosphorescent methods and antibody assays such as enzyme linked immunosorbant assay (ELISA) or radioimmunoassay (RIA).

In one embodiment, the results of the quantitative analysis of each fraction are used to prepare a quantitative

fingerprint of the sample. The fingerprint is composed of the quantity of component in each of the marker fractions and the identity of the component. This quantitative fingerprint is then compared to the known standard fingerprint which has been established (FIG. 1) in order for the material to be considered as pharmaceutical grade. If the quantitative fingerprint of the sample falls with the range of quantities sat forth for the pharmaceutical grade fingerprint, then the material may be identified as being of pharmaceutical grade.

As a further part of the quality assurance assay, the individual marker fractions may be subjected to biological assays. The biological assays which are used to test the various fractions are the same as those used for the standard fingerprint and will also depend upon the particular clinical use intended for the material.

The bioactivity fingerprint generated for the material is compared to the standard bioactivity fingerprint which has been established in order for the material to be considered as pharmaceutical grade. If the bioactivity fingerprint of the sample falls within the range of bioactivities set forth for the pharmaceutical grade fingerprint, then the material is identified as, and approved as, being of pharmaceutical grade.

5.1.2. ALTERNATIVE METHODS OF DEVELOPING A PHARMAPRINT

The method of pharmaprinting a botanical when the putative active components are not known also begins with a literature review. It involves reviewing any chemical literature, biological literature, published bioassays or clinical data available for the botanical or for botanicals with related activities. Based on the disease state, a series of relevant bioassays is chosen. The activity of the total sample or extract is analyzed in the bioassays. Those bioassays that show activity are then used to analyze fractions of the botanical for which the putative active components are not yet known. The fractionation is based on the usual methods, *e.g.*, separation by dielectric constant,

polarity, size or absorptive power. The fractions are then analyzed to determine which fraction is responsible for the activity. Assuming activity is found, each active fraction is refractionated to isolate the individual putative active components, *i.e.*, pure chemical compounds. Based on knowing the individual chemical compounds and knowing their quantitative biological activity, a quantitative potency curve may be drawn and the 50% inhibitory concentration (IC_{50}) for each individual chemical component may be determined. If the putative active components are agonists, then other parameters (binding, activation, response) will be needed. In the general case, the bioassay will consist of appropriate tests of the stimulatory or inhibitory effects of the constituents, fractions or entire extract, followed by an appropriate quantitative evaluation of those effects. For the most likely (or typical) assays in which a standard (or radiolabelled) agonist causes a measurable effect, inhibition by the subject material may be assessed and expressed typically via the determination of an IC_{50} value, or other suitable measure (*e.g.*, K_i). The activities of individual putative active components are then totalled and that summation is compared to the activity in the unfractionated botanical sample. If these components account for a substantial portion of the activity, then one has an initial fingerprint of "active components" for the botanical where the active components were not known.

5.1.3 ADDITIONAL VARIATIONS ON THE METHOD OF DEVELOPING A PHARMAPRINT

The general method outlined above for Pharmaprinting a botanical whose putative active components are not known has several variations should complications arise in the course of the analysis. One variation occurs when the summation of individual components do not account for a substantial portion of the biological activity of the botanical. At this point there are several likely reasons for the reduced activity of the individual components, one, decomposition or

degradation of active components or, two, a synergistic effect. In another possible scenario there may be no significant activity seen from any of the fractions, but the whole botanical or extract shows activity in the bioassay.

To determine if the active components are decomposing in the course of the assay is relatively simple. One merely recombines all of the fractions and compares the activity of the recombined fractions with the activity of the crude material. If substantial activity has been lost, then the problem is probably decomposition. To determine which active components may be decomposing, the chromatographic analysis of the crude botanical is compared with that of the recombined fractions. Peaks that are missing or are reduced in size indicate that components are decomposing. To overcome decomposition many methods exist. Typically, milder extraction/fractionation methods such as liquid-liquid chromatography (counter-current chromatography) or supercritical carbon dioxide extraction or chromatography may be used.

Another explanation for the activity of the individual fractions not accounting for a substantial portion of the expected total activity is a synergistic effect between one or more active components. To determine that a synergistic effect is taking place, pair-wise recombined fractions need to be analyzed. If the combined fractions show more activity than the individual fractions, two or more individual components in the fractions may be acting synergistically. For example, if you have three fractions each responsible for 10% of the bioactivity (their uncombined additive bioactivity is 30%) but the combined fractions are responsible for 100% of the activity. In that case the fractions are acting synergistically. By repeated pair-wise recombination of fractions or looking at larger fractions, any synergistic activity will be discovered. Once two fractions show synergy, they are then refractionated as above, and pairs of individual fractions or pairs of isolated components are studied to find the individual components that act

synergistically. Three way comparisons of individual components or fractions may also be studied.

What if the fractions have no activity in the bioassay in which the botanical shows activity? Here, the explanations include decomposition, synergy, or many many active components such that no individual fraction shows activity. The first step would be to fractionate each initial fraction and see if active components appear in the bioassay. It that does not succeed, the fractions should be recombined and assayed to determine if decomposition of the actives is taking place. If decomposition is taking place, the appropriate measures as described above should be taken. If there is no decomposition, then alternative methods of fractionation should be tried. Eventually, large enough or appropriately sized or selected fractions will show activity. If synergy is a suspected problem, then proceed as in the synergy section described above.

5.2. TYPES OF BOTANICALS MATERIALS

The examples of different types of botanical materials which may be processed in accordance with the present invention include any botanical material including but not limited to material from higher plants. Examples of botanicals may be found in the Leung and Foster, 1996; Bisset, 1994; or Tyler and Foster, 1996 references. Specific examples of botanicals exemplified in the detailed description include the following: Agnus Castos, Aloe, Asian Ginseng, Bilberry, Black Cohosh, Chamomile, Chestnut, Coriolus Versicolor, Echinacea, Evening Primrose, Feverfew, Garlic, Ginger, Ginkgo, Goldenseal, Green Tea, Guggulipid, Hawthorn, Ivy, Kava, Licorice, Milk Thistle, Mistletoes (American, Asian and European varieties), Passion Flower, Pumpkin, Pygeum, Rosemary, Siberian Ginseng, Saw Palmetto, St. John's Wort, Stinging Nettle and Valerian. Additionally combinations of one or more botanical materials such as Eschinacea and Goldenseal, may be processed in accordance with the present invention.

5.2.1. METHODS OF PROCESSING AND EXTRACTING BOTANICAL MATERIALS

The botanical material may be processed to form an aqueous or organic extract of the whole plant or a selected part of the plant. The botanical material can also be processed in whole or part to form a powder. Many of the botanicals of interest are commercially available as powders, aqueous extracts, organic extracts or oils. In one embodiment, extracts of the plant material are preferred because they are easier to dissolve in liquid pharmaceutical carriers. However, powdered plant materials are well-suited for many applications where the drug is administered in solid form, *e.g.*, tablets or capsules. Such methods are well known to those of skill in the art. Furthermore, many of the plant materials and/or extracts are available commercially. As examples of the processing and extracting of botanicals the following examples are provided. Additional examples are provided in the detailed description.

For a typical root, it may be sliced, frozen or pulverized. If powdered it is then shaken with an appropriate solvent and filtered (Tanabe et al., 1991, *Shoyakugaku Zassi*, 45(4):316-320). Alternatively, the following methods are used: the root is homogenized, acetone extracted and filtered; the botanical may be steam distilled to obtain essential oils and the distillate dissolved in acetone-water or appropriate solvent; or the cut rhizomes are frozen and/or freeze-dried and the resulting powder acetone-water extracted (Tanabe et al., 1991, *Shoyakugaku Zassi* 45(4):321-326). Another method of processing botanicals is aqueous extraction with 100°C water (Yamahara et al., 1985, *J. Ethnopharmacology* 13:217-225). The initial solvent extract from the methods above may be further extracted using liquid/liquid extraction with an appropriate solvent. The botanical may be extracted in two steps using polar and non-polar solvents respectively. The solvents are then evaporated and the fractions combined (Nagabhusan et al., 1987, *Cancer Let.* 36:221-233). Botanicals may also be

processed as a paste or powder which may be cooked (Zhang et al., 1994, *J. of Food Science* 59(6):1338-1343).

A variety of solvents may be used to extract the dried botanicals, for example acetone, acetonitrile, dichloromethane, ethanol, isopropanol and supercritical carbon dioxide (Sipro et al., 1990, *Int. J. of Food Science and Technology* 25:566-575 and references therein).

For other botanicals such as Saw Palmetto, the medicinal products are the seed oil or dried berries. In a typical preparation, a hexane or supercritical carbon dioxide extract is prepared. Many preparations are commercially available, for example Permixon™ or Talso™. For an example of supercritical carbon dioxide extraction of a botanical, see Indena, European Patent No. 0 250 953 B1. Alternatively, the botanical may be crushed and extracted with an appropriate solvent (90%) in a soxhlet (Elghamry et al., 1969, *Experienia* 25(8):828-829). The botanical may also be ethanol extracted (Weisser et al., 1996, *The Prostate* 28:300-306).

The dried material may be prepared in a variety of ways of freeze-drying including drying via microwave, cooling with liquid nitrogen and pulverizing; drying at 70°C under vacuum for a duration of 10 hours; or air-drying in the shade (List and Schmidt, Hagers Handbuch der Pharmazeutischen Praxis, Springer-Verlag:New York, 1993, 1973-79; Araya et al., 1981, *Journal of Comparative Pathology*, 135-141). Teas, dilute aqueous extracts, also known as decoctions, may be made in 60-80°C water (Nosel and Schilcher, 1990). Extraction is more efficient when the particle size is less than .25 mm (List and Schmidt, Phytopharmaceutical Technology, CRC Press:Boca Raton, FL, 1989).

Various guidelines a available for preparing oil extracts of botanicals. e botanical may be digested (macerated) in oil at 45°C for 10 days, while others recommend 70°C for 12-24 hours (Hobbs, 1989, *HerbalGram* 18/19:24-33; Smith et al., Quality Validation Laboratory - Herb Pharm: Williams, OR, 1996). In St. John's Wort for example, exposing the preparation to sunlight during the

extraction process has been reported to result in a four-fold increase in flavonoid content calculated as quercetin (Maisenbacher and Kovar, 1992). Additionally, for St. John's Wort, two-fold increases of hypericin have been reported in oil preparations in which the material has been further extracted with alcohol, and mixed with the oil (Georgiev et al., 1983, *Nauchni Tr.-Vissh Inst. Plovid.* 30:175-183).

Alternatively an alcohol-water preparation may be prepared of the botanical (Dyukova, 1985, *Farmitsiya* 34:71-72; Georgiev et al., 1985, *Nauchni Tr.-Vissh Inst. Plovid.* 32:257-263; Wagner and Bladt, 1994, Kowalewski et al., 1981, *Herba Pol.* 27:295-302). According to Hagers Handbuch a tincture of a botanical, such as St. John's Wort, may be prepared by using drug or freezing ethanol soaked, and filtering and preserving in dark bottles (List and Hörhammer, 1993).

Some botanicals, such as St. John's Wort, are both temperature and light sensitive. For this type of botanical the material should be dry packed with a refrigerant or shipped under refrigeration and protected from light and air. In St. John's Wort, hypericin content has been shown to drop significantly in powdered extract, tablet and juice preparations when stored at temperatures of 60°C for more than six weeks (140°F). Dry extracts stored at 20°C were found to remain stable for at least one year (Adamski et al., 1971, *Farm. Pol.* 27:237-241; Benigni et al. Hypericum. Piante Medicinali: Chimica, Farmacologia e Terapia, Milano: Inverni & Della Beffa; 1971). Other St. John's Wort constituents, hyperforin and adhyperforin found in oil preparations are highly unstable, especially when exposed to light, and can degrade in as little as 14 days (Meisenbacher et al., 1992, *Planta Med.*, 351-354). Stability (in absence of air) was increased to six months in a preparation extracted with ethanol. Similarly, up to four xanthones and several flavonoids including quercetin and I3', II8-biapigenin have been detected suggesting these may be among

the active constituents in external preparations (Bystrov et al., 1975, *Tetrahedron Letters* 32:2791-2794).

5.3 SEPARATION OF FRACTIONS

Once the sample extract has been prepared and/or alternatively purchased as a commercially available extract, a portion needs to be subjected to fractional analysis. If the fingerprint has already been established, the sample or aliquot is separated into the same plurality of marker fractions which are present in the standard fingerprint. Each of the marker fractions will include one or more of the active or inactive components. The marker fractions are established on an individual basis for each botanical material being tested. For some materials only a few marker fractions are required. For other more complex materials, there may be numerous marker fractions. For example in mistletoe, *Viscum album* protein extract, the preferred protein marker fractions are those fractions which are separated based on the sugar binding affinity of the fraction. However, different parameters for identifying and separating the materials into the marker fractions may be established based upon the types of components present in the botanical material. Separation of the sample into the marker fractions may be accomplished by any of the conventional separation techniques including liquid chromatography and extraction procedures. The same procedures which were used to establish the standard fingerprints should be used. Since the various fractions may be tested for biological activity, it is preferred that non-destructive separation techniques be utilized. Liquid column chromatography is a useful separation technique with affinity chromatography based on the specific binding ability of the compounds (*e.g.*, carbohydrates and target enzymes) being particularly used.

After the fractionation, the solvent is removed and the material is dissolved in an appropriate medium for the bioassays. Examples of appropriate media include DMSO, ethanol, various detergents, water and an appropriate buffer.

The choice of solvent will depend on the chemical nature of the component being analyzed and the compatibility with the assay system.

5.4 ESTABLISHMENT OF APPROPRIATE BIOASSAYS

Exemplary biological assays may include any cell proliferation assays, such as the measurement of L 1210 cell inhibition, immune activity or inhibition of critical enzyme which relates to specific diseases. Examples of other transformed cell lines which can be used for bioassays include HDLM-3 Hodgkin's lymphoma and Raji Burkitt's lymphoma, hepatoma cell line, primary or established cultures of human/animal cell lines which carry specific cell receptors or enzymes.

The results of the biological assays are used to prepare a bioactivity fingerprinting of the material. The fingerprint can be as simple as an assay of two selected marker fractions. Conversely, the fingerprint can include numerous different bioassays conducted on numerous different fractions. The same assay may be conducted on different marker fractions. Also, different assays may be conducted on the same marker fraction. The combination of bioassays will depend upon the complexity of the given botanical material and its intended clinical use. The bioassays will be the same as those conducted in establishing bioactivity fingerprint of the standard material.

5.4.1. ENZYMATIC AND RECEPTOR BASED ASSAYS

Enzymatic and receptor based assays are preferable in the practice of this invention. Assays are chosen either based on accepted enzymatic assays for a clinical disorder or they are chosen from relevant assays for a given clinical disorder. It is important to choose appropriate bioassay. Ideally, a bioassay should be rugged, that is reproducible over time and show a quantitative dose response over a wide concentration range. An issue faced with a botanical for which the active components are not known is the choice of a

relevant bioassay. Here, the human therapeutic use will serve as a guide to pick assays known in the art based on possible mechanisms of action. The mechanism of action should be consistent with a clinically relevant endpoint. There are a wide array of clinically relevant assays based on enzymatic activity, receptor binding activity, cell culture activity, activity against tissues and whole animal *in vivo* activity.

This section will address enzymatic and receptor binding assays. There are many books on enzymatic or receptor assays, for example, Methods in Enzymology by Academic Press or Boyers, The Enzymes. "Bioactive Natural Products, Detection, Isolation, and Structural Determination", S. M. Colegate and R. J. Molyneux, CRC Press (1993), also discusses specific bioassays. "Methods in Cellular Immunology", R. Rafael Fernandez-Botran and V. Vetvicka, CRC Press (1995) describes assays from immune cell activation and cytokine receptor assays. "Screening Microbial Metabolites for New Drugs-Theoretical and Practical Considerations" describes additional methods of finding pharmaceutical (Yarbrough et al. (1993) *J. Antibiotics* 46(4):536-544). There are also many commercial contract research vendors, including Panlabs™ and Novascreen™.

For example, for a botanical useful for treating neurological disorders, the array of bioassays might include adrenergic receptors, cholinergic receptors, dopamine receptors, GABA receptors, glutamate receptors, monoamine oxidase, nitric oxide synthetase, opiate receptors, or serotonin receptors. For cardiovascular disorders the array of assays may include adenosine A_1 agonism and antagonism; adrenergic α_1, α_2, β_1 agonism and antagonism; angiotensin I, inhibition; platelet aggregation; calcium channel blockade; ileum; cardiac arrhythmia; cardiac inotropy; blood pressure; heart rate; chronotropy; contractility; hypoxia, hypobaric; hypoxia, KCN; portal vein, potassium depolarized; portal vein, spontaneously activated; or thromboxane A_2, platelet aggregation. For metabolic disorders the following bioassays

may be used: cholesterol, serum HPL, serum total; serum HPL/cholesterol ratio; HDL/LDL ratios; glucose, serum - glucose loaded; or renal function, kaluresis, saluresis, and urine volume change. For allergy/inflammation disorders the following bioassays may be used: allergy, passive cutaneous anaphylaxis; bradykinin B_2; contractility, tracheal; histamine H_1 antagonism; inflammation, carrageenan; leukotriene D_4 antagonism; neurokinin NK_1 antagonism; or platelet activating factor, platelet aggregation. For gastrointestinal disorders the following bioassays may be used: cholecystokinin CCK_A antagonism; cholinergic antagonism, peripheral; gastric acidity, pentagastrin; gastric ulcers, ethanol; ileum electrical stimulation modulation; ileum electrical stimulation spasm or serotonin 5-HT_3 antagonism. For antimicrobial, antifungal, or antitrichomonal disorders the following are used: *Candida albicans; Escherichia coli; Klebsiella pneumonaie; Mycobacterium ranae; Proteus vulgaris; Pseudomonas aeruginosa; Staphylococcus aureus*, methicillin resistant; *Trichomonas foetus*; or *Trichophyton mentagrophytes*. For other indications, one of skill in the art would be able to choose a relevant list of bioassays.

Specific examples of assays based on enzymes or receptors include the following: aldol-reductase; angiotensin converting enzyme (ACE); rat androgen receptor; CNS receptors; cyclooxygenase 1 or 2 (Cox 1, Cox 2); DNA repair enzymes; dopamine receptors; estrogen receptors; fibrinogen; GABA A or GABA B; β-glucuronidase; lipoxygenases, *e.g.*, 5-lipoxygenase; monoamine oxidases (MAO-A, MAO-B); platelet activating factor (PAF); prostacyclin cyclin; prostaglandin synthetase; serotonin assays, *e.g.*, 5-HT activity or other serotonin receptor subtypes; serotonin re-uptake activity or thromboxane synthesis activity. Specific enzymatic assays are available from a variety of sources including Panlabs™ Inc (Bothell, WA) and Novascreen™ (Baltimore, MD). Additional assays include: ATPase inhibition, benzpyrene hydroxylase inhibition, HMG-CoA reductase inhibition, phosphodientrase inhibition, protease

inhibition, protein biosynthesis inhibition, tyrosine hydroxylase inhibition, testosterone-5α-reductase and cytokine receptor assays.

5.4.2 CELL CULTURE AND OTHER ASSAYS

In addition to the enzymatic assays, there are also other biological assays. Preferably, these assays are performed in cell culture but may be performed on the whole organism. Cell culture assays include activity in cultured hepatocytes and hepatomers (for effect on cholesterol levels, LDL-cholesterol receptor levels and ratio of LDL/HDL cholesterol); anti-cancer activity against L 1210 cells, modulating receptor levels in PC12 human neuroblastoma cells; cell culture activity of luteinizing hormone (LH), follicle stimulating hormone (FSH) or prolactin; Ca^{2+} influx to mast cells; cell culture assays for phagocytosis, lymphocyte activity or TNF release; platelet aggregation activity or activity against HDLM-3 Hodgkin's lymphoma and Raji Burkitt's lymphoma cells, antimitotic activity, antiviral activity in infected cells, antibacterial activity (bacterial cell culture) and antifungal activity. Tissue or whole animal assays may also be used including anti-inflammatory mouse ear dermatitis, rat paw swelling; muscle contractility assays, vasodilation assays; or whole animal carbon clearance tests. These assays are available from a variety of sources including Panlabs™ Inc. (Bothell, WA).

5.4.3. ANTICANCER ACTIVITY

The anticancer effects of drug can be studied in a variety of cell culture systems; these include mouse leukemias, L 1210, P388, L1578Y etc. Tumor cell lines of human origin like KB, and HeLa have also been used. In a typical assay tumor cells are grown in an appropriate cell culture media like RPMI-1640 containing 10% fetal calf serum. The logarithmically growing cells are treated with different concentrations of test material for 14-72 hours depending upon cell cycle time of the cell line. At the end of the

incubation the cell growth is estimated by counting the cell number in untreated and treated groups. The cell viability can be ascertained by trypan blue inclusion test or by reduction of tetrazolium dyes by mitochondrial dehydrogenase. The ability of a drug to inhibit cell growth in culture may suggest its possible anticancer effects. These effects can be verified in animals bearing tumors, which are models for human disease (Khwaja, T.A., et al. (1986) *Oncology*, 43 (Suppl. 1): 42-50).

The most economical way to evaluate the anticancer effects of an agent is to study its effects on the growth of tumor cells in minimum essential medium (MEM) containing 10% fetal calf serum. The drug-exposed cells (in duplicates) are incubated in a humidified CO_2 incubator at 37 °C for 2-4 days, depending upon the population-doubling time of the tumor cells. At the end of the incubation period the cells are counted and the degree of cell growth inhibition is calculated from a comparison with untreated controlled cells grown under identical conditions. The type of cell lines used have varied from laboratory to laboratory depending upon individual needs. The National Cancer Institute (NCI) in the United States recommends the use of KB cells (a human nasopharyngeal carcinoma) for the evaluation of anticancer drugs *in vitro*. The cell growth inhibition is determined by estimating the protein content (Lowry's method) of the drug-treated and untreated controls. NCI has also recommended the use of suspension culture of mouse leukemia P388 for the evaluation of anticancer potential of plant extracts and related natural products.

We have routinely used mouse leukemia L1210 cells, cultured in microtiter plates for *in vitro* assays for anticancer activity. The cell population-doubling time of leukemia L1210 is 10-11 h and a drug exposure of 48 h (3-4 generations of logarithmic growth) is used for the evaluation of its antitumor activity. For growth inhibition studies reported here, all stock solutions and dilutions were made with sterile 0.9% NaCl solution. The cell cultures were

seeded at 2-5 x 10^4 cells/ml in duplicates for each inhibitor concentration in a microtiter place (0.18 ml/well). The inhibitors were added in 0.02 ml volume to achieve 1:10 dilutions in each case. The covered microtiter plate was incubated for 48 h in a humidified CO_2 incubator containing 5% CO^2 in air. At the end of the incubation period aliquots of each well were added to a measured volume of isotonic saline and counted in an electronic cell counter. Because mistletoe extract at high concentrations caused rapid cellular fragmentation, the test microtiter plates were routinely checked under microscope prior to cell number counting so that the results were not compromised. The cell viability was determined by trypan blue exclusion. The results were calculated by plotting percent cell growth inhibition (as compared to cell density of the saline-treated controls) versus log of drug concentration and expressed as the concentration which caused 50% inhibition (ID_{50}) in cell growth as determined from the graph.

The cytotoxic effects of a drug on a tumor cell line can also be evaluated but these experiments need a longer period of time and are more expensive. In these studies drug-treated cells are washed free of drug and then plated in soft agar or an appropriate medium and the cellular viability is estimated by the ability of the surviving cells to multiply and form microscopic colonies. The number of cellular colonies obtained with certain drug concentrations is compared with those obtained from untreated controls to evaluate cell kill or cytotoxic activity. In our studies with mistletoe extract we have used suspension cultures of EMT-6 cells (a mouse mammary adenocarcinoma). These cells were grown in Eagle's MEM (F14) containing 10% dialyzed fetal calf serum and antibiotics. The cell suspension was spun and the pellet suspended in Spinner's medium supplemented with 10% dialyzed fetal calf serum (70 cells/ml), plated in plastic Petri dishes and incubated for 2 h to permit cells to attach. At this time cells were exposed to various concentrations of extract for 2-24 h. Then, the medium was

removed and replaced with drug-free medium and the dishes incubated for 5-7 days. The colonies were stained with methylene blue (0.33% in 0.01% KOH) and counted with an automatic colony counter. The plating efficiency of EMT-6 cells was 46%. (Khwaja et al., 1986, *Oncology*, *43 (Supp. 1)*:4250).

5.4.4. ANTIVIRAL ACTIVITY

The antiviral activity of different drugs can be ascertained in cell culture of human cell lines like HeLa or H9 lymphoma cells. These cells are infected with virus and the virus is allowed to propagate in cell cultures. The ability of virus to produce cell lysis or cytopathic effects is taken to as end point. Thus HIV infection of H9 cells carries production of multinucleated cells. The cytopathic effects, if reduced or eliminated by certain concentrations of the drug, will point to its potential as anti-HIV agent. These results can be validated by estimation of viral enzyme in the cell cultures, *e.g.*, by studying the amount of the expression of viral reverse transcriptase. A decreased expression of the viral enzyme would support antiviral effect of the drug treatment (Khwaja, T.A. U.S. Patent No. 5,565,200; J. Levy et al. 1984, *Science* 225: 840).

5.5. ANALYTICAL METHODS FOR ANALYZING CHEMICAL COMPONENTS

There are many methods to separate and analyze individual chemical components including gas chromatography (GC), mass spectroscopy (MS), GC-MS, high pressure liquid chromatography (HPLC), HPLC-MS, thin layer chromatography (TLC), gel chromatography and reverse phase chromatography (RPC). These chromatographic methods may be performed either on an analytical scale or a preparative scale. To determine the actual chemical structure of unknown components, nuclear magnetic resonance (NMR) and mass spectrum fragmentation analysis are typically used.

The determination of the type of chromatography will depend on the chemical components most likely responsible for the bioactivity. For example if the bioactivity is likely due to fatty acids, the fatty acids are esterified and the esters analyzed on a GC. For organic compounds with alcohol groups, they are modified to prepare ethers, silyl derivatives or other less polar functional groups. These derivatives are then suitable for analysis by GC (Steinke et al., 1993, *Planta Med.* 59:155-160; Breu et al., 1992, *Arzneim.-Forsch/Drug Res.* 42(1):547-551). If the activity is most likely due to flavonoids, HPLC is the method of choice. Reverse-phase HPLC (RP-HPLC) has been used to analyze flavonoids from a variety of botanicals, specifically Hawthorn, passion flower, chamomile, ginkgo (Pietta et al., 1989, *Chromatographia* 27(9/10):509-512). Plant constituents have been quantitatively determined by TLC (Vanhaelen and Vanhaelen-Fastre, 1983, *J. Chromatography* 281:263-271) as well as MS-analysis for garlic. CRC Handbooks of Chromatography on "Analysis of Lipids", K. D. Mukherjee, "Analysis and Characterization of Steroids", H. Lamparczyk, J. Sherma, and "High-Performance Liquid Chromatography of Peptides and Proteins", C.T. Mant and R.S. Hodges, are available and describe columns and solvent systems.

5.6. ANALYSIS OF FRACTIONS

In an alternative embodiment, rather than basing the fingerprint on discrete chemical components of known bioactivity, one may also establish a fingerprint based on defined fractions or classes of compounds. Some chemical constituents in botanicals form such a complex mixture of closely related components that from a practical point of view it requires to base the fingerprint on fractions or classes of components further than indivisual components. Examples of these types of components are lectins (sugar-binding proteins) or glycoproteins. There are many examples of fractional analysis (Gel Filtration Principles and Methods

Pharmacia Biotech, Rahms i Lund: Sweden; Utsumi et al., 1987, *J. Biochem.* 101:1199-1208.

5.7. AN ILLUSTRATIVE EXAMPLE OF A PHARMAPRINTED BOTANICAL DRUG

The following example sets forth the general production of pharmaceutical grade extracts of mistletoe which are effective in the treatment of AIDS and certain cancers. Initially the extract was separated into different classes of its chemical entities (components). It was found that the major biological activity was associated with its protein fraction (>95%) and the residual activity was separated in the alkaloid fraction. The quantitative fingerprint is based on the measurement of specific protein fractions which have different binding affinities for various sugars. The standard pharmaceutical grade quantitative fingerprint requires that the fractions contain between 0.01 and 1.0 mg/ml of Ca^{++} dependent sugar-binding proteins which are capable of binding with lactose, galactose, melibiose, N-acetyl-D-galactosamine or fucose and exhibit an inhibitory concentration of below about 0.50 pg/ml. "Inhibitory concentration", as used in this section is a measure of the sugar-binding protein's ability to inhibit the *in vitro* growth of certain cancerous cell lines. The inhibitory concentration is expressed in μg of sugar-binding protein per ml of extract solution which is required to cause a 50% inhibition of the growth of a particular cancerous cell line. The preferred cell line which is used to measure inhibitory concentrations is a leukemia cell line identified as L1210. This particular cancerous cell line is available from a number of commercial sources. L1210 calls have been used in the past as a screening system for testing drug efficacy. Details regarding the culturing and growth of L1210 cell are described in a number of scientific articles (*Oncology* 43:42-50 and *Experientia* 1980, 36:599-600). Other cell lines which can be used to determine inhibitory concentrations include KB cells or other rapidly growing cell lines which demonstrate

repeatable results. Other parameters like inhibition of macromolecule synthesis in a given cell culture line may be used.

Identification of the mistletoe extract as pharmaceutical grade preferably requires that the standard fingerprint of the extract must also contain one or more Ca^{++} non-dependent sugar-binding protein fractions which are capable of binding with lactose, galactose, melibiose, N-acetyl-D-galactosamine, or fucose. The quantitative level of the Ca^{++} non-dependent sugar-binding proteins must be between about 0.1 and 2.0 mg/ml for each fraction. The inhibitory activity of the one or more Ca^{++} non-dependent sugar-binding protein fraction is preferably below about 0.5 μg/ml.

5.7.1. PLANT SOURCE/EXTRACTION METHOD

The mistletoe powder (processed biological material) is prepared according to any of the known powdering procedures. Any type of mistletoe may be used; however, to maintain the rejection rate of extracts at a low level, it is preferred that the mistletoe extract be prepared from the *Viscum album*, coloratum species, which is found on oak trees in South Korea or the Viscum album L. species, which is commonly found throughout Europe. Further, it is preferred that the mistletoe be flash frozen shortly after harvesting and then ground to a powder in the frozen state. Flash freezing with liquid nitrogen or similar cryogenic liquid is preferred. The entire mistletoe plant may be used in preparing the powder. The preferred harvesting time is when the berries are ripe. Due to the variable and relatively unknown nature of all of the complex components in mistletoe, it is preferred that all of the above listed preferred steps be followed in order to optimize the number of extract preparations which meet the requirements of the method of the present invention. The fresh soft part of the mistletoe plant (including leaf, stem and/or berries) may be pressed to squeeze the cell-sap, which, on dilution with water or

sterile physiological saline, will provide an aqueous extract.

The mistletoe powder is extracted using a substantially pure aqueous solution. The extracting solution may include additional components to enhance the extraction process. The extract may include a salt such as calcium chloride or sodium chloride in an amount sufficient to enhance extraction of proteins from the mistletoe sap. Salt concentrations on the order of 0.02 M are preferred. Detergents such as TRITON® x-100 may also be added to enhance extraction of protein from the plant cell walls. The extractant is preferably buffered with 0.02 M Tris to a pH of about 7.8. The extraction procedure should utilize an amount of extractant relative to the amount of mistletoe being extracted which will produce levels of proteins as sat forth below. Preferably, between about 100 and 400 grams of mistletoe powder will be extracted with between about 1000 and 4000 ml of aqueous solution. The powder is mixed in the solution and left to stand for from one to four hours. The powder particulates remaining after the extraction period are separated by filtration, centrifugation or other conventional separation technique to produce the aqueous mistletoe extract.

5.7.2. FRACTIONAL ANALYSIS

The mistletoe extract, prior to pharmacological analysis of its contents, is preferably assayed for its overall biological activity. The biological evaluation of the extract is carried out on the basis of its inhibition of the growth of mouse leukemia L1210 in culture. From this initial test, the IC_{50} (concentration in μg/ml of the extract which will cause 50% inhibition in the growth of L1210 cells as compared to untreated controls) of the extract is determined. The biological activity of the extract is expressed in terms of "activity units" (A.U.), which represent the ratio of concentration of the extract (expressed in μg extract/ml) to its IC_{50} value (μg/ml). To pass this initial step of extract evaluation, 1 ml of 1 % extract (10 mg total extract) must

contain 100 or more activity units. Extracts with lower than 100 A.U. values are rejected, and those with a passing grade are further analyzed to establish specific values and ratios of its biologically active components as described below.

The separation of the mistletoe extract into its various protein fractions is preferably conducted by affinity column chromatography as schematically represented in FIG. 10. The separation is preferably conducted in two stages in order to separate the proteins which require Ca^{++} for binding to sugar and those proteins which are non-Ca^{++} dependent. A buffer which contains ethylene diamine tetraacetic acid (EDTA) or other chelating agent is used to elute the Ca^{++} dependent sugar-binding proteins from affinity columns which have been treated with the specific sugars of interest, *i.e.* lactose, galactose, melibiose, N-acetyl-N-galactosamine, and fucose. The resulting fractions are then analyzed for quantitative protein content. Other affinity columns based on similar principles of chromatography may also be used, *e.g.* mannose, rhamnose, maltose, asialofeutin, glucosamine, and n-acetyl glucosamine. The appropriate levels and activities of the lectins in the extract which are specific for these sugars can be established by measuring their levels in extracts which meet the criteria for sugar-specific lectins set forth above.

After elution of each of the sugar-specific affinity columns with EDTA or EGTA (ethylene glyceryl tetracetic acid)-containing buffer, the columns are then eluted with buffers that each contain the sugar which corresponds to the sugar bound to the respective affinity columns. Sepharose® 4B is a preferred column material. The various fractions resulting from this second elution contain the non-Ca^{++} dependent sugar-binding proteins. The protein content of each of these fractions is also quantitatively determined. Protein quantitation can be performed using any of the conventional quantitative analytical procedures including ninhydrin based tests, spectroscopic determination and Bio-Rad or Pierce analysis. The use of multiple buffers as

described above is preferred so that the total level of each type of protein is determined. Affinity column chromatography is preferred because it is a conventional separation technique which is well-known to those of ordinary skill in the art and which is well-suited for separating the proteins from the extract based on their sugar-binding specificity. Other separation procedures may be utilized provided that an accurate measurement of each of the selected sugar-binding proteins is provided.

An alternate method for analyzing the lectin (sugar-binding protein) content, alkaloidal fraction and viscotoxin levels in the extract involves the use of ammonium sulfate. In this method, the total proteins of mistletoe preparation are precipitated with 70% ammonium sulfate. The preparation is centrifuged and the precipitate, which contains a mixture of the viscotoxins and the sugar-binding proteins (lectins), is separated on an acid-hydrolysed Sepharose 4B column. The bound lectins can be eluted from the column with buffers containing specific sugars as described above. Affinity chromatography to identify the specific sugar-binding proteins can be carried out as also described above. The unbound proteins which initially fan through the column contain viscotoxins which are quantitated according to conventional protein quantitative analysis.

The ammonium sulfate-supernatant, which contains the alkaloidal fraction, is lyophilized and the alkaloids are extracted with chloroform. The chloroform residue provides the total alkaloids which are also quantitated by weighing or another conventional method.

The five Ca^{++} dependent and non-Ca^{++} dependent sugar-binding protein fractions which are used to generate fingerprints are those that bind with lactose, galactose, melibiose, N-acetyl-D-galactosamine, or fucose. The concentration of each of the sugar binding proteins (both Ca^{++} dependent and non-Ca^{++} dependent) is between 0.1 and 2.8 weight percent of the total protein content of the extract. The specific relative weight percent ranges which are

necessary for an extract to meet the requirements of the present invention are set forth in Table 1. Tables 2 and 3 set forth the weight percent ranges for pharmaceutical grade extracts prepared from European and Korean mistletoe, respectively.

The relative percentage of each sugar-binding protein with respect to the total protein content of the extract will remain fairly constant irrespective of extraction conditions. However, the actual concentration levels of the various sugar-binding proteins will vary in each extract depending upon a number of factors including the relative amounts of mistletoe and aqueous extractant, the length of extraction and temperature. It is preferred that the extract be analyzed to determine the concentration levels of the various specified sugar-binding proteins and that these concentration levels be used as the primary method of determining whether an extract meets the pharmaceutical grade requirements of the present invention. However, the extract may be diluted or concentrated to achieve protein concentration levels outside the preferred concentration ranges provided that the relative percentages of the sugar-binding proteins remain within the limits set forth in Table 1, for general mistletoe extracts, and Tables 2 and 3, for European and Korean mistletoe extracts, respectively. The extract, once it has been identified as pharmaceutical grade, may be dehydrated and stored as a powder for rehydration and used at a later time to treat AIDS or cancer.

TABLE 1

GENERAL QUANTITATIVE FINGERPRINT
PHARMACEUTICAL GRADE MISTLETOE EXTRACT

	Percentage By Weight of Total Protein in Extract
(Ca^{++} dependent sugar-binding proteins)	
1. Lactose	0.1 - 2.3
2. Galactose	0.1 - 0.9
3. Melibiose	0.1 - 0.6
4. N-Acetyl-D-galactosamine	0.1 - 1.6
5. Fucose	0.1 - 1.3
(Non-Ca^{++} dependent sugar-binding proteins)	
1. Lactose	0.1 - 2.4
2. Galactose	0.1 - 1.9
3. Melibiose	0.1 - 2.2
4. N-Acetyl-D-galactosamine	0.1 - 2.8
5. Fucose	0.1 - 2.0

TABLE 2

QUANTITATIVE FINGERPRINT FOR PHARMACEUTICAL GRADE EUROPEAN MISTLETOE EXTRACT

	Percentage By Weight of Total Protein in Extract
(Ca^{++} dependent sugar-binding proteins)	
1. Lactose	1.2 - 2.3
2. Galactose	0.1 - 0.9
3. Melibiose	0.1 - 0.6
4. N-Acetyl-D-galactosamine	0.5 - 1.6
5. Fucose	0.3 - 1.3
(Non-Ca^{++} dependent sugar-binding proteins)	
1. Lactose	1.4 - 2.4
2. Galactose	0.9 - 1.9
3. Melibiose	1.2 - 2.2
4. N-Acetyl-D-galactosamine	0.8 - 2.8
5. Fucose	1.0 - 2.0

TABLE 3

QUANTITATIVE FINGERPRINT FOR PHARMACEUTICAL GRADE KOREAN MISTLETOE EXTRACT

	Percentage By Weight of Total Protein in Extract
(Ca^{++} dependent sugar-binding proteins)	
1. Lactose	0.1 - 0.5
2. Galactose	0.1 - 0.5
3. Melibiose	0.1 - 0.5
4. N-Acetyl-D-galactosamine	0.1 - 0.5
5. Fucose	0.1 - 0.5
(Non-Ca^{++} dependent sugar-binding proteins)	
1. Lactose	0.1 - 0.5
2. Galactose	0.1 - 0.8
3. Melibiose	0.1 - 0.5
4. N-Acetyl-D-galactosamine	0.1 - 0.7
5. Fucose	0.1 - 0.5

The preferred concentration level of the Ca^{++} dependent sugar-binding proteins in the extract is within the range of about 0.01 to 1.0 mg/ml. The method of the present invention can be carried out by measuring only one of the Ca^{++} dependent sugar-binding proteins to establish if the extract is within pharmaceutical grade limits. However, it is preferred that two or more of the sugar-binding protein levels be measured, *e.g.* galactose, lactose and/or N-acetyl-D-galactosamine specific proteins. Even more preferably, the extract is analyzed to determine if the concentration levels of all five Ca^{++} dependent sugar-binding proteins meet the above quantitative fingerprint limits. The method can also be carried out by measuring various combinations of 3 or 4 of the specified Ca^{++} dependent sugar-binding proteins. Concentration ranges and inhibitory activity ranges are set forth in Tables 4, 5 and 6.

TABLE 4

QUANTITATIVE AND BIOACTIVITY FINGERPRINTS FOR PHARMACEUTICAL GRADE MISTLETOE EXTRACT

	Concentration (mg/ml)	Inhibitory Concentration (IC_{50})
(Ca^{++} dependent sugar-binding proteins)		
1. Lactose	0.1 - 0.5	0.01 - 0.5
2. Galactose	0.01 - 0.5	0.001 - 0.5
3. Melibiose	0.01 - 0.6	0.001 - 0.6
4. N-Acetyl-D-galactosamine	0.1 - 1.6	0.01 - 1.6
5. Fucose	0.1 - 1.3	0.01 - 1.3
(Non-Ca^{++} dependent sugar-binding proteins)		
1. Lactose	0.1 - 0.5	0.0001 - 0.5
2. Galactose	0.1 - 2.0	0.001 - 0.5
3. Melibiose	0.1 - 0.5	0.001 - 0.6
4. N-Acetyl-D-galactosamine	0.1 - 1.0	0.001 - 1.6
5. Fucose	0.1 - 1.5	0.01 - 1.3

TABLE 5

QUANTITATIVE AND BIOACTIVITY FINGERPRINTS FOR PHARMACEUTICAL GRADE EUROPEAN MISTLETOE EXTRACT

	Concentration (mg/ml)	Inhibitory Concentration (IC_{50})
(Ca^{++} dependent sugar-binding proteins)		
1. Lactose	0.1 - 0.3	0.01 - 0.1
2. Galactose	0.01 - 0.1	0.0001 - 0.01
3. Melibiose	0.01 - 0.1	0.001 - 0.01
4. N-Acetyl-D-galactosamine	0.1 - 0.3	0.01 - 0.1
5. Fucose	0.5 - 1.0	0.01 - 0.1
(Non-Ca^{++} dependent sugar-binding proteins)		
1. Lactose	0.1 - 0.3	0.001 - 0.1
2. Galactose	0.1 - 0.3	0.01 - 0.1
3. Melibiose	0.1 - 0.3	0.1 - 0.5
4. N-Acetyl-D-galactosamine	0.05 - 0.3	0.001 - 0.01
5. Fucose	0.05 - 0.3	0.1 - 0.5

TABLE 6

QUANTITATIVE AND BIOACTIVITY FINGERPRINTS FOR PHARMACEUTICAL GRADE KOREAN MISTLETOE EXTRACT

	Concentration (mg/ml)	Inhibitory Concentration (IC_{50})
(Ca^{++} dependent sugar-binding proteins)		
1. Lactose	0.1 - 0.5	0.1 - 0.5
2. Galactose	0.2 - 0.6	0.1 - 0.5
3. Melibiose	0.1 - 0.5	0.1 - 0.5
4. N-Acetyl-D-galactosamine	0.5 - 1.0	0.1 - 0.5
5. Fucose	0.1 - 0.5	0.1 - 0.5
(Non-Ca^{++} dependent sugar-binding proteins)		
1. Lactose	0.1 - 0.5	0.0001 - 0.009
2. Galactose	1.0 - 2.0	0.001 - 0.01
3. Melibiose	0.1 - 0.5	0.001 - 0.01
4. N-Acetyl-D-galactosamine	0.5 - 1.0	0.001 - 0.1
5. Fucose	1.0 - 2.0	0.001 - 0.1

The concentration level of the non-Ca^{++} dependent sugar-binding proteins in the extract must be within the range of about 0.10 to 2.0 mg/ml. The method of the present invention can be carried out by measuring only one of the non-Ca^{++} dependent sugar-binding proteins to establish if the extract is within pharmaceutical grade limits. However, as above, it is preferred that two or more of the non-Ca^{++} dependent sugar-binding protein levels be measured. Even more preferably, the extract is analyzed to determine if the concentration levels of all five non-Ca^{++} dependent sugar-binding proteins meet the required concentration limits. The most preferred method involves determining the complete protein finger print, *i.e.* the protein concentration in all ten sugar-binding protein fractions. In this most preferred embodiment, the extract is not identified or otherwise considered to be a pharmaceutical grade extract unless all ten protein fractions have the required concentration levels

set forth above. The standard fingerprint parameters are set forth in Tables 4, 5 and 6.

The bioactivity of the various protein fractions is used in combination with their respective concentration levels to identify the extract as pharmaceutical grade in accordance with the present invention. The various proteins which make up each of the Ca^{++} dependent sugar-binding protein groups must each exhibit an inhibitory concentration of between about 0.001 and 0.5 μg/ml. The proteins which make up each of the non-Ca^{++} dependent sugar-binding groups must also each exhibit an inhibitory concentration of between about 0.0001 and 0.5 μg/ml.

The method for measuring inhibitory action is set forth in numerous scientific articles including the references mentioned previously. It is preferred that the inhibitory action be measured *in vitro* with respect to leukemia L1210 cells. This procedure is preferred because L1210 cells are readily available, are easily maintained by well-known culturing procedures, and provide consistently reproducible results. The inhibitory concentration of each sugar-binding protein fraction is determined by adding increasing amounts of the fraction and determining when cell growth is inhibited by 50% as compared to a control culture. It is preferred that both the concentration level and the inhibition concentration of each of the sugar-binding proteins be measured and that they all be within the ranges set forth above and in Tables 4, 5 and 6 in order for the extract to be identified as pharmaceutical grade in accordance with the present invention.

Once the concentration levels and/or inhibitory concentration of the designated sugar-binding proteins has been established, the extract is identified as pharmaceutical grade if the above limits are met. If one or more requirements are not met, *i.e.*, the sample fingerprint does not match with the standard fingerprint, the extract is rejected. The extracts which are identified as pharmaceutical grade are then used in treatment programs for

treating diseases such as AIDS and cancer. The pharmaceutical grade extracts are not only useful in treating AIDS, but may be used to treat any individual with a suppressed immune system. If the extract has protein levels which are above the limits set forth above, the extract may be diluted as required to bring the extract protein concentrations down to the established limits. If the extract protein levels are below the limits, the extract is rejected and not identified as pharmaceutical grade.

It is preferred that the extract be initially screened for overall activity before beginning the more rigorous analysis of the sugar-binding protein fingerprint. It was discovered that extracts which do not meet certain total activity levels will also not meet the more specific protein fingerprint requirements of the present invention. The activity units are determined in the same manner as for the individual protein fraction(s) with the only difference being that the entire extract is being tested. In accordance with the present invention, the extract must have and activity of greater than 100 AU. Table 7 sets forth the results of initial screening wherein a number of different mistletoe extracts were screened to determine their biological activity using the L1210 cells as previously described. As can be seen, commercial preparations such as ISCADOR do not meet the initial screening test and therefore are not pharmaceutical grade in accordance with the present invention. The ISCADOR extracts also do not meet the more stringent specific protein fingerprint requirements of the present method. However, the extracts n-T4GEN and T4GEN, which do meet the specific protein concentration fingerprint of the present invention, both have activity levels well above the minimum of 100 A.U. The screening procedure is preferred because it allows non-pharmaceutical grade extracts to be identified relatively quickly without having to conduct the more time consuming protein fingerprinting. Once an extract passes this initial screening step, it then still must meet the further protein fingerprint requirements in order to qualify as

pharmaceutical grade in accordance with the present invention.

TABLE 7

BIOLOGICAL ACTIVITY OF MISTLETOE EXTRACTS IN TERMS OF ACTIVITY UNITS (A.U.)

Sample Identity	Activity units/ml, (1% Extract)
1. ISCADOR M, Arg., 10%	80
2. n-T4GEN, 40%	400
3. T4GEN, 5%	227
4. ISCADOR M, 5%	30
5. ISCADOR M, 20%	37
6. T4GEN, 1%	140
7. T4GEN, 10%	217
8. ISCADOR M, 20%	26
9. n-T4GEN, 40%	416
10. n-T4GEN	192
11. n-T4GEN	811

Activity unit per ml = $\frac{\text{concentration of sample in } \mu g/ml}{IC_{50}\ (\mu g/ml)}$

It is also preferred that the fraction of the extract which contains protein that does not bind to sugar be analyzed to provide a further fingerprint that is used in identifying whether the extract is pharmaceutical grade. As shown in FIG. 9, a fraction containing unbound protein remains after separation of the sugar-binding proteins from the extract. This unbound protein fraction contains an "alkaloid" fraction and a viscotoxin fraction. These two fractions can be isolated from each other by column chromatography using a Sephadex G-75 column or equivalent. The amount of alkaloids in the alkaloid fraction should be about 10 to 50 μg per ml of 1 percent extract. The amount of viscotoxin in the protein fraction should be about 10 to 40 μg per ml of 1 percent extract. As previously described, a 1 percent extract is one where 1 gram of total initial non-dehydrated plant material is extracted with 100 ml of aqueous extractant. The amount of viscotoxins and alkaloids will

increase proportionally as the amount of plant material extracted per 100 ml increases.

In use, the extracts may be used as is or diluted with suitable pharmaceutical carriers and administered according to known procedures for treating AIDS or a particular cancer. For treating AIDS, the extract is preferably injected subcutaneously in doses ranging from 0.01 to 1 ml of a 1 percent extract. Extracts which are more concentrated, such as 2 and 5 percent extracts, may be used. Extracts with even higher concentrations may also be used depending upon the dosage required. The injections are preferably given twice a week, but may be given more often. For cancerous tumors, the extract is injected directly into the tumor or may be injected subcutaneously.

5.7.3. BIOLOGICAL ACTIVITY ANALYSIS

An extract identified herein as n-T4GEN was tested to demonstrate its anti-HIV activity. n-T4GEN was analyzed and found to meet the sugar-binding protein fingerprint set forth in Table 6. The amount of protein in the alkaloidal and Viscotoxin fractions was also found to be within the required fingerprint ranges. The n-T4GEN extract was prepared from Korean mistletoe. n-T4GEN was added to culture wells in an amount sufficient to provide a concentration of 1 A.U. of a 1 percent extract per ml of test solution (equivalent to 10 μg of extract per ml of test solution). This concentration of n-T4GEN inhibited HIV-induced cytopathic effects in H9 lymphoid human leukemia cells with concomitant reduction in viral reverse transcriptase levels in the infected cells.

Human immunodeficiency virus (HIV) infects T4 lymphocytes. In the H9 human lymphoma cell line, the virus produces giant multinucleated syncytial cells. After 3-6 days of viral infection, the number of syncytia correlates with the degree of virus growth as quantified in the presence and absence of the drug being tested. These cytopathic effects and assay of viral reverse transcriptase were used to demonstrate anti-HIV effects of n-T4GEN.

The anti-HIV assay using 1 μg/ml of a 1 percent n-T4GEN extract per ml of test solution was conducted as follows:

HIV inoculum was standardized for reverse transcriptase (RT) activity using purified avian myeloblastosis viral RT (BRL Labs, Gaithersberg, MD) and used to infect polybrene treated H9 cells at 0.02 RT units of HIV per 2 x 10^6 cells. The virus was adsorbed for 2 hours at 37°C and then the cells were washed twice and resuspended in RPMl 1640 containing 10% fetal bovine serum at 2 x 10^5 cells/ml and dispersed in 1 ml aliquots into 24-well plates (Falcon Division, Becton Dickinson Co., Cockneyville, MD). Syncytial giant cell formation appeared at 5-6 days post infection, and this cytopathic effect (CPE) was quantitatively measured by dispersing 0.1 ml aliquots into 0.1 ml absolute methanol and enumerating the giant cells microscopically. The inhibition of CPE by antiviral treatment with 1 A.U. (10 μg) of a 1 percent n-T4GEN extract per 1 ml of test solution was compared to untreated, infected H9 cells.

Reverse transcriptase activity was measured using 1 ml culture aliquots which were clarified at 600 x g for 30 minutes, precipitated in 10% polyethylene glycol - 0.13 M NaCl at 4°C for 18 hours, and centrifuged at 600 x g for 60 minutes. The pellet was dissolved in glycerol-Tris buffer (50% glycerol, 25 mM Tris HCl pH 7.5, 5 mM dithiothreitol, 15 mM KCl, 0.025% Triton-X, and 0.25 mM EDTA). The RT assay was adapted from published methods (Levy et al., 1984, *Science* 225: 840; Ho et al., 1984, *Science* 226:451), using a final reaction mixture containing 40 mM Tris-HCl pH 7.8, 2.2 mM dithiothreitol, 10 mM $MgCl_2$, 50 mM KCl, 0.03% Triton-X, 25 μCi ^{3}H-thymidine triphosphate (New England Nuclear, Boston, MA), and 50 pg/ml poly rA oligo ^{dT}12-18 (BRL, Gaithersburg, MD), Background counts were determined using poly dA oligo ^{dT}12-18 as template and subtracted from the poly rA dT primer cpm to determine the thymidine incorporation specifically due to RT-activity.

The results of the tests are set forth in Table 8 as follows:

TABLE 8

Effects of Mistletoe Extract on the Infectivity of HIV to H9 Lymphoma Cells in Culture

n-T4GEN* (μg/ml)	Cytopathic (Syncytia cells) Day 5	Reverse Transcriptase (cpm) Day 10
0.01	++	97,896 (100)
0.10	++	77,974 (79)
1.00	++	65,932 (67.3)
10.00	±	32,128 (32.8)
100.00	(toxic)	4,200

H9 lymphoma cells growing in RPMI-1640 media containing 10% fetal calf serum were infected with HIV (1000,000 RT counts) on day 1 and various concentrations of n-T4GEN extract. On day 5 cells were observed for cytopathic effects (syncytia), and on day 10 assayed for RT activity. (++) Denotes extensive giant cells, (±) fewer syncytial cell.

* Amounts expressed as μg of 1 percent extract per ml of cell culture.

The results show that n-T4GEN at non-toxic concentrations inhibited HIV-induced cytopathic effects on H9 lymphoma cells. At these concentrations (10 μg/ml) there was also a significant (67.2%) inhibition of the viral reverse transcriptase. An extract identified herein as T4GEN was tested along with n-T4GEN to demonstrate their anti-cancer activity. T4GEN was analyzed and found to meet the sugar-binding protein fingerprint set forth in Table 5. The amount of protein in the alkaloidal and viscotoxin fractions were also found to be within the required fingerprint ranges. The T4GEN extract was prepared from European mistletoe.

Anticancer activities of T4GEN and n-T4GEN were studied in animals bearing subcutaneous transplants of C3H mammary adenocarcinoma 16/C. This tumor is maintained as a lung passed tumor in C3H female mice. In this example, tumors (1 x 10^5 cells) were transplanted (S.C.) in 18-20 g B6C3F1 hybrid female mice. On the following day, the tumor bearing animals were randomized and separated into different treatment groups (10 mice per group). There were 15 animals in the control group who received only physiological saline during the treatment periods. The treatments (i.p.) were started 48

hours after the transplants and given for a duration of 14 days (daily single injections). Animals were weighed on days 5, 9 and 14 to assess toxic effects. Tumors were measured on days 21 and 28 post transplants and the results are represented as tumor weights using formula $(l \times w^2)/2$ (l = length of tumor, w = width of tumor expressed in mm).

The results set forth below show that the T4GEN extract at a dose schedule equivalent to 1 ml of a 1 percent extract/kg (20 mg/kg), qd (1-14) caused a 98% inhibition in the growth of this tumor. In the same experiment the n-T4GEN (5mg/kg, qd 1-14) caused 33% inhibition of growth of mammary adenocarcinoma 16/C, however, 30% of the treated animals remained tumor free until termination of the experiment (day 93). This animal model is an accepted model for human breast carcinomas. The results are set forth in Table 9 as follows:

TABLE 9

Effect of T4GEN and n-T4GEN on the Growth of Subcutaneous Transplants of C3H Mammary Adenocarcinoma 16/C in B6C3F1, Female Mice

Treatments i.p.	▲ Wts (g) (day 14)	Tumor Wts (day 21)	Tumor Weight Inhibition %	Tumor Free Animals (day 93)
1. Saline, Controls	+0.21	0.27	—	1/15
2. T4GEN*				
1 ml/kg, qd(1-14)	+0.82	0.15	44	0/10
2 ml/kg, qd(1-14)	-2.12	0.01	98	0/10
3. n-T4GEN*				
.25 ml/kg, qd(1-14)	+1.33	0.15	44	1/10
.50 ml/kg, qd(1-14)	+0.73	0.18	33	3/10
4. 5-Fluorouracil 98 mg/kg, qd(1,7,14,21)	+1.77	0.00	100	0/6

* Amounts expressed as ml of 1 percent extract.

5.8. METHODS OF USE OF PHARMAPRINTED MATERIALS

After the botanical material has an established fingerprint, individual samples are then analyzed to determine if they fall within the accepted standards. Once the sample has been approved it is suitable for a variety of diseases relevant to humans and animals. Such materials are useful in clinical trials so as to provide materials of consistent quality and precise dose formulations for trials. The pharmaprinted material is also useful for toxicological tests in animals where once again the consistency of the material is useful for quantitative toxicological analysis. In many cases it would be of use as a reference material for analytical or biological use.

The pharmaprinted botanical materials are useful for any disease state for which a botanical drug is associated. See for example Leung and Foster, 1996 and Herbal Drugs and Phytopharmaceuticals, 1994. More specific examples of disease states or therapeutic indications include AIDS, mild-to-moderate depression, anti-arthritic, anti-cancer, anti-diarrhetic, anti-inflammatory, anti-nausea via GI, anti-rheumatic, anti-spasmodic, anti-ulcer, antibacterial, antimutagenic, antioxidant, antiviral, arthritis, asthma, blood pressure, benign prostatic hyperplasty (BPH), bronchial asthma, bronchitis, calmative, cerebral circulatory disturbances, cholesterol lowering, cirrhosis, dermatological anti-inflammatory, diabetes, diuretic, drastic cathartic, dysmenorrhea, dyspepsia, environmental stress, expectorant, free radical scavenger, GI distress, hemorrhoids, hepatitis, hepatoprotective, hyperlipidemia, hyperprolactinemia, immunomodulatory activity, increase fibrinolysis, resistance to bacterial infection, inflammation, insomnia, lactation, liver protection, longevity, menstrual cycle regulation, migraine, muscle pain, osteoarthritis, pain, peripheral vascular disease, platelet aggregation, PMS, promote menstrual flow, prostatic disorders, reduce triglycerides, relieve menstrual pain, respiratory tract infections (RTI), retinopathy, rheumatism, sedative, sleep-promoting agent,

sore throat, stimulate hair growth, superficial wound healing, tinnitus, topical eczema (dermatitis), urinary tract infection (UTI), varicose veins, venous insufficiency or wound healing.

The following examples are presented for purposes of illustration only and are not intended to limit the scope of the invention in any way.

6. EXAMPLE: SAW PALMETTO, *Serenoa repens, Serenoa serrulata, Sabal serrulata*

6.1. PLANT SOURCE/EXTRACTION METHOD

The Saw Palmetto is a small palm indigenous to the United States. In botanical preparations, the small brownish-colored berries have been used for many years to treat diseases of the bladder and the prostate. Extracts of the Saw Palmetto are prepared in variety of ways, typically, hexane extraction or supercritical carbon dioxide extraction. The extraction methods are described above which describes methods of processing and extracting botanical materials. There are also lipidic extracts and saponifiable extracts that are commercially available from Madaus, S.A.

6.2. COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are many commercial suppliers of *Sabal serrulata* extracts. The following names are used: IDS 90 (Weisser et al., 1996, *The Prostate* 28:300-306); Strogen Forte, Talso™, SG290 Talso™ uno, commercially available from Sanofi Winthrop GmbH (Munich, Germany). Extracts are also available from Madaus S.A. (Köln, Germany). Permixon™ is available from Centre de Recherches P. Fabre (Castres, France). Several varieties of Permixon extract are available, including a lipophilic extract, LSESr extract, and a PA109 extract. Another product available is Prostaserene™, a purified extract of *Serenoa repens*, commercially available from Therabel Pharma™, (Belgium). Saw Palmetto is also produced by the following companies: NaturaLife, Herbal Choice, Botalia Gold, Herb Pharm, PhytoPharmica.

6.3. CLINICAL USE FOR ALLEVIATION OF THE SYMPTOMS OF BENIGN PROSTATIC HYPERTROPHY

In Europe, botanical materials represent nearly half of all prescriptions for benign prostatic hypertrophy (BPH) (Di Silverio et al., 1993, *Minerva Urol. Nefrol.* 45:143-9) (Di Silverio et al.). Some of the more common plant extracts, or phytotherapeutics, prescribed for BPH are obtained from: *Serenoa repens* (Saw Palmetto Berry), *Pygeum africanum* (Plum Bark), and *Cucurbita pepo* (Pumpkin Seed). By far the most widely used botanical material for the treatment of BPH is a lipid extract of Saw Palmetto berries. Saw Palmetto Berry extract (SPB-extract) is nontoxic and in extensive clinical trials has demonstrated little or no adverse side effects. The most extensive clinical trials of SPB-extract were conducted in France. In these trials the SPB-extract Permixon was used. This extract has been commercially available since 1982. According to French scientists, Permixon is very safe, and there is little evidence of undesirable side effects. Today, Permixon and a variety of other products are available in over twenty countries throughout the world.

A number of clinical studies with SPB-extracts have been published in recent years. In 1984, Champault et al. reported a double blind, plácebo controlled study in 110 outpatients (Champault et al., 1984, *Br. J. Clin. Pharm.* 18:461-462). In this study, 55 patients received the SPB-extract Permixon (160 mg, twice daily) and 55 patients received placebo treatment for 30 days. The study reports statistically significant improvements in nocturia, intensity of dysuria, urine flow rate, and post-nicturition residue. This report records minor side effects (*e.g.*, headaches) in 5 patients. Di Silverio et al. have reported on the treatment of 34 BPH patients with the SPB-extract Strogen Forte (160 mg, twice daily) for three months (Di Silverio et al., *supra*). The results of the study showed subjective improvements in 60% of patients and included a reduction of urine volume in 50%, a slight reduction in prostate volume in

53%, and significant increase in serum testosterone levels and reduction in intra-prostatic DHT concentrations. The authors have also reviewed studies during 1983-1985 which report efficacy and tolerability of the SPB-extract Strogen Forte in BPH patients. In 1993, Romics et al. reported on a one year treatment study in 42 patients (Romics et al., 1993, *Internat. Urol. and Nephrol.* 25(6):565-569). This study reported significant improvements in objective symptoms like night urination (68.4%) residual volume (74.7%) and interrupted urine stream and post-urination dribbling in 80% of patients. No side effects were reported. In another study, Vohlensieck et al. reported a 12-week treatment study of 1334 BPH outpatients with the SPB-extract of *Sabal serrulata* (Vohlensieck et al., 1993, *Fortsch der Med.* 111(18):323-326). Under this treatment, the volume of residual urine decreased by 50%, pollakisuria decreased on the average by 37%, and nocturia by 54%. The number of patients with dysuria pain decreased from 75% to 37%. Furthermore, they found the efficacy of the drug "good to excellent" in more than 80% of the cases and tolerability "good to excellent" in more than 95% of the patients.

Thus, Saw Palmetto is useful to treat and/or ameliorate BPH and/or urinary dysfunction.

Other researchers have reported combination treatments. Specifically, SPB-extract used in combination with extracts from pumpkin seeds (53 patients) (Carbin et al., 1990, *Br. J. Urol.* 66:639-641) or urica extract (2,080 patients) (Schneider et al. 1996, *Fortsch der Med.* 113(3):37-40). All of these studies support the efficacy of SPB-extract in the treatment of BPH and/or urinary dysfunction.

6.4. FRACTIONAL ANALYSIS OF SAW PALMETTO

The fractionation of the contents of commercially available gel caps of Saw Palmetto was performed using normal phase flash chromatography. This method was selected as a preferable prep-chromatographic technique on the basis of observed excellent mass recovery (>90%), the separation of

the selected standards (fatty acids, their esters), as well as separation of the other co-ocurring components. A detailed description of the materials and methods utilized is described below.

A comprehensive search of the literature on Saw Palmetto (*Sereno repens*) indicated that phytosterols (β-sitosterol), fatty acids (palmitic, oleic, linoleic, linolenic, myristic and lauric acids), as well as their ethyl esters, are the components of Saw Palmetto with the most consistent bioactivity in a number of assays [fatty acids/esters: 5α-reductase (Weisser, 1996, *supra*), androgen receptors (Casarosa, 1988); phytosterols (especially β-sitosterol, although less than 10% of the activity of estradiol): estrogenic activity (Duke, 1985)].

Prep-CC Method: Approximately 5 g of the contents of a commercial gel capsule of Saw Palmetto contents were dissolved in 25 ml of CH_2Cl_2. The resultant extract was loaded onto a preparative flash glass chromatography column prepacked with SiO_2. The flash CC (column chromatography) conditions were as follows: column-60μm SiO_2; each mobile phase volume = two column volumes; ten fractions were collected with the elution profile shown in the table below. The total recovery of 4.77 grams gave a 92% total mass balance.

Analytical capillary GC Method: The GC system was a Varian GC/FID equipped with Star data handling software. Separations were made using 1μl injection volumes injected onto a capillary column (Restek RTX-2330, 30 m column, 0.25 μm, 0.20 μmdf) and a temperature gradient system as follows: 55°C for 1 min.; 7.5°C/min. to 260°C, hold for 5 min. The column flow rate was kept at 1.0 ml/min., with the injection split ratio 1:100. Injector and detector temperatures used were 260°C. There are many sources of standards for the Saw Palmetto assay, including Aldrich Chemical Co., Inc. (Milwaukee, WI, USA).

Approximately 5 grams of commercially available SPB-extract (from gel capsules) were dissolved in 25 ml of

methylene chloride. Flash chromatography was performed using this solution. The chromatography system consisted of 60 μm Silica Gel and the following eluting solvents:

Mobile Phase	Hexane	Acetone	MeOH (% V/V)
1	100	0	0
2	90	10	0
3	80	20	0
4	70	30	0
5	60	40	0
6	50	50	0
7	40	60	0
8	30	70	0
9	20	80	0
10	0	0	100

The amount of dried residue recorded from each fraction of mobile phase collected was as follows 1 (~0.1 g), 2 (~2.5 g), 3 (~1.6 g), 4 (~0.1 g), 5 (~0.1 g), 6-9 (~0.0 g), and 10 (~0.5 g). The total dried residue recovered via flash chromatography was calculated to be 4.77 grams. Given the initial load of extract was 5.15 grams, 92% of the extract was recovered as dried residue.

6.5. BIOLOGICAL ACTIVITY ANALYSIS

Based on a literature review and according to the teaching of the method of the present invention, the following categories of bioassay were chosen to assess biological activity of Saw Palmetto for BPH *in vitro*: anti-androgenic, anti-inflammatory, cyclooxygenase/lipoxygenase (CO/LO) inhibition, muscle contractility. From these categories, the specific assays studied were as follows: 5-lipoxygenase assay, cyclooxygenase-1-assay, cyclooxygenase-2 assay (Panlabs, WA), and an androgen receptor assay developed at the Medical School of Georgia as described below.

6.5.1. RAT PROSTATIC ANDROGEN RECEPTOR ASSAY: COMPETITION NUCLEAR RECEPTOR LIGAND BINDING ASSAY AGAINST DIHYDROTESTOSTERONE

6.5.1.1 MATERIALS AND METHODS

Animals

Male rats were obtained at 60 days of age (300-350 g body weight) from Harlan (St. Louis, MO) and allowed to acclimate for 24 hours to a air-conditioned, light controlled room with a 12 hour light-dark cycle. Rats were fed with Purina chow and tap water, *ad libitum*.

All animals studies carried out were approved by the Medical College of Georgia Institutional Committee for the Care and Use of Animals in Research and Education in accordance with the guidelines of the National Institutes of Health and United States Department of Agriculture.

Steroid and reagents

General chemicals (reagent grade), free fatty acids, fatty acid ethyl esters and radioinert steroids were obtained from Sigma Chemical Company (St. Louis, MO). ^{3}H-dihydrotestosterone (5α-androstan-17β-ol-3-one; 60 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). 100% ethanol was used throughout for the preparations of all inhibitors and radioinert chemicals.

Androgen Receptor Binding

The animals were treated intraperitoneally with testosterone (400 μg/100 g body wt). After 1 hour, the animals were sacrificed by decapitation and the ventral prostates were rapidly removed and placed in ice-cold "homogenization buffer" (10 mM Tris-HCl, 1.5 mM Na_2EDTA, 0.5 mM dithiothreitol, 0.25 M sucrose, 1 mm phenylmethylsulfonylfluoride, pH 7.4 at 22°C). The prostates were minced and homogenized on ice with a Polytron homogenizer (set at 4) using 10 second bursts alternated with a 30 second cooling period at a tissue-buffer ratio of 550 mg/ml. The homogenate was centrifuged at 800 g for 20

minutes at 4°C. The nuclear pellet was then resuspended in ice-cold "nuclear buffer" (10 mM Tris, 0.5 mM dithiothreitol, 0.25 M sucrose, 2.5 mM $MgCl_2$, pH 7.4 at 22°C, 550 mg tissue/ml). The resuspended pellet was homogenized on ice using a glass Dounce homogenizer until suspension became uniform.

Aliquots of the nuclear suspension obtained after rehomogenization of the nuclear pellets were dispensed into 12 x 75 mm glass test tubes containing 10 µl of ^3H-dihydrotestosterone with or without 5 µl of various concentrations of inhibitors in a final volume of 1 ml. Non-specific binding was determined using radioinert dihydrotestosterone (10^{-5} M) in place of the inhibitor. The test tubes were incubated for 20 hours at 15°C.

After an overnight incubation, 1 ml of ice-cold nuclear buffer was added followed by centrifugation at 800 g for 10 minutes at 4°C. The nuclear pellets were washed 3 times by resuspension in 1 ml of the same ice-cold nuclear buffer, with mixing and centrifugation as above. After discarding the final supernatant, 1 ml of 100% ethanol was added to each pellet and then vortexed. Test tubes were placed in a 30°C water bath for 40 minutes, with vortexing every 10 minutes and a final centrifugation at 800 g for 10 minutes. The ethanol extracts were decanted into vials containing 4 ml of scintillation fluid (Ecoscint A), shaken and counted.

Compounds, extracts and fractions were screened at an initial concentration of 1 X 10^{-5} and in some cases at 2 X 10^{-5}. If an activity of greater than 50% inhibition was observed at 2 X 10^{-5} or less, a full dose response curve was carried out. The results of this analysis are shown in the summary table for Saw Palmetto. K_{50} displacements for auric acid ester, Linoleic acid ester and extract #3 are shown in Figs. 4, 5 and 6 respectively.

The results, expressed as percent inhibition of ^3H-Dihydrotestosterone binding, at two concentrations of putative active components, of the rat prostatic androgen receptor assay are below in the summary table.

6.5.2. 5-LIPOXYGENASE ASSAY

5-lipoxygenase catalyzes the oxidative metabolism of arachidonic acid to 5-hydroxyeicosatetraenoic acid (5-HETE), the initial reaction in the biosynthetic pathway leading to the formation of the leukotrienes. The procedure was as follows. 5-lipoxygenase assays were run using a crude enzyme preparation from rat basophilic leukemia cells (RBL-1). Test compounds were pre-incubated with the enzyme for 5 minutes at room temperature and the reaction was initiated by addition of substrate (arachidonic acid). Following an 8 minute incubation at room temperature, the reaction was terminated by addition of citric acid, and levels of 5-HETE were determined by 5-HETE radioimmunoassay (RIA). Compounds are screened at 30 μM (Shimuzu et al., 1984, *Proc. Natl. Acad. Sci. USA* 81:689-693).

The following reference compounds were used for the inhibition of 5-lipoxygenase: reference compounds, (IC_{50} (μM)): BW-755C, (6.6); nordihydroguaiaretic acid (NDGA), (0.26); phenidone, (30).

Compounds and fractins were screened at an initial concentration of 3 X 10^{-5}. If an activity of greater than 50% inhibition was observed at 3 X 10^{-5}, a full dose response curve was carried out. The results of this analysis are shown in the summary table for Saw Palmetto.

6.5.3. CYCLOOXYGENASE-1 ASSAY

Cyclooxygenase-1 (from ram seminal vesicles), 125 units per assay tube, was pre-incubated with 1 mM GSH, 1 mM hydroquinone, 1.25 mM hemoglobin and test compound for 1 minute at 25°C. The reaction was initiated by addition of arachidonic acid (100 mM) and terminated after 20 minutes incubation at 37°C by addition of trichloroacetic acid (TCA). Following centrifugal separation and addition of thiobarbiturate, cyclooxygenase activity was determined by reading absorbance at 530 nm (Evans et al., 1987, *Biochem. Pharamac.* 36:2035-2037; Boopathy and Balasubramanian, 1988, *J. Biochem.* 239:371-377).

The following reference compounds were used for the inhibition of cyclooxygenase 1: reference compounds, (IC_{50} (μM)); aspirin, (240); indomethacin, (1.7).

Compounds and fractions were screened at an initial concentration of 3 X 10^{-4}. If an activity of greater than 50% inhibition was observed at 3 X 10^{-4}, a full dose response curve was carried out. The results of this analysis are shown in the summary table for Saw Palmetto.

6.5.4. CYCLOOXYGENASE-2 ASSAY

Cyclooxygenase-2 (from sheep placenta), 80 units per assay tube, was pre-incubated with 1 mM GSH, 1 mM hydroquinone, 1.25 mM hemoglobin and test compound for 1 minute at 25°C. The reaction is initiated by addition of arachidonic acid (100 mM) and terminated after 20 minutes incubation at 37°C by addition of TCA. Following centrifugal separation and addition of thiobarbiturate, cyclooxygenase activity is determined by reading absorbance at 530 nm (Boopathy and Balasubramanian, 1988; Evans et al. 1987; O'Sullivan et al., 1992, *Biochem. Biophy. Res. Commun.* 187:1123-1127).

The following reference compounds were used for the inhibition of cyclooxygenase-2: reference compounds, (IC_{50} (μM)): aspirin, (660); indomethacin, (2.4).

The results of the cyclooxygenase-2 assay are below in the summary table.

SUMMARY TABLE
Saw Palmetto Extract - Biological Assay Results

Component/Extract Fraction	Androgen Receptor	Cox1	Cox2	5-Lipo
Lauric Acid	Negative	Negative	Negative	Negative
Linoleic Acid	Negative	Negative	Negative	Negative
Linolenic Acid	Negative	233uM	Negative	12 uM
Myristic Acid	Negative	Negative	Negative	Negative
Oleic Acid	Negative	Negative	Negative	Negative
Palmitic Acid	Negative	Negative	Negative	Negative
Lauric Ester	130 nM	Negative	Negative	Negative
Linoleic Ester	6 um	Negative	Negative	Negative
Linolenic Ester	Negative	Negative	Negative	Negative
Myristic Ester	Negative	Negative	Negative	Negative
Oleic Ester	Negative	Negative	Negative	Negative
Palmitic Ester	Negative	Negative	Negative	Negative
Extract #1	Negative	Negative	Negative	Negative
Extract #2	30 nM*	Negative	Negative	Negative
Extract #3	3.5 uM	Negative	Negative	Negative
Extract #4	Not tested	Negative	Negative	Negative
Extract #5	Negative	Negative	Negative	Negative
Fraction # 1	Not tested	Negative	Negative	Negative
Fraction # 2	Negative	Negative	Negative	Negative
Fraction # 3	Negative	Negative	Negative	Negative
Fraction # 4	Not tested	Negative	Negative	Negative
Fraction # 5	Not tested	Negative	Negative	Negative
Fraction # 10	Not tested	Negative	Negative	Negative
Fraction 6-9**	Not tested	Not tested	Not tested	Not tested
Beta-Sitosterol	60% @ 10uM	Not tested	Not tested	Not tested

* See Discussion in The Pharmaprinting of Saw Palmetto section
**Dry Residue zero grams

6.5.5. 5-ALPHA-REDUCTASE ASSAYS

6.5.5.1. PREPARATION OF PROSTATIC 5-ALPHA-REDUCTASE FROM RATS

In a typical experiment, adult male Sprague-Dawley rats (10-20) are sacrificed by cervical dislocation. The prostates are removed and cleaned by removal of connective tissues. The tissues are maintained at 0-4°C and suspended in a 3-fold volume of buffer A (20 mM sod. phosphate pH 6; 0.32 mM sucrose; and 0.1 mM dithiothreitol), cut, minced, and homogenized with a Polytron homogenizer. The homogenate is centrifuged at 10,000 g for 60 minutes and 10,000 g supernatant is centrifuged at 140,000 g for 60 minutes. The two pellets are combined and suspended in twice the pellet volume of buffer B (sod. phosphate 2 mM, pH 6.5; 2 M NaCl; digitonin 5 mg/ml; 0.1 mM EDTA, 40% glycerol and 1 mM dithiothreitol) at 0°C for 60 minutes. The suspension is centrifuged at 150,000 g for 60 minutes and the supernatant containing solubilized 5-alpha-reductase after addition of 5 mM NADPH is estimated for its protein contents and stored at -70°C as 5-alpha-reductase.

6.5.5.2. PREPARATION OF PROSTATIC 5-ALPHA-REDUCTASE FROM HUMANS

Human prostatic tissue is obtained from BPH patients undergoing surgical transvesical resections. The tissue is transported to the laboratory in ice cold saline within 60 minutes. The tissue samples are cleaned, chopped into 1-3 g pieces, and quick frozen in liquid nitrogen and stored at -70°C. BPH is confirmed by histological examination.

The prostatic tissues are thawed and cut with fine scissors (or pulverized at liquid N_2 temp.) and homogenized with one 30 second burst of sonicator at 4°C in a 5-fold volume of buffer (100 mM Tris/HCl pH 7.4, 20% glycerol, 100 mM sodium citrate, 100 mM KCl, 1mM EDTA and 15 mM β-mercaptoethanol). The homogenate is filtered through glass wool to remove cell debris and then centrifuged at 800 g for 10 minutes to provide a nuclear pellet. The supernatant is

divided into 1 ml aliquots to be centrifuged at 120,000 g for 45 minutes to provide microsomal pellets containing 5-alpha-reductase which are stored at -70°C or suspended in buffer B containing 0.25 mg/ml Lubrol PX or 0.5 mg/ml digitonin and passed through a 25 gauge syringe to make a homogenate which is centrifuged at 120,000 g for 45 minutes. The supernatant microsomal 5-alpha-reductase (estimate protein contents) is used in assays or stored in 40% glycerol at -20°C without loss of activity.

6.5.5.3. ASSAY OF 5-ALPHA-REDUCTASE ACTIVITY

The 5-alpha-reductase assays are studied by following the reduction of radiolabelled [^{3}H] testosterone (T) to [^{3}H] 5-α-dihydrotestosterone (DHT) at 37°C. Tubes (duplicates) with 1 ml Tris-HCl (pH 7.4) buffer containing 100 mM sodium citrate, 100 mM KCl, 20% (v/v) glycerol, 1 mM EDTA, 15 mM β-mercaptoethanol, 5 mM glucose-6-phosphate dehydrogenase and 1 μM-[^{3}H] testosterone, are preincubated at 37°C for 15 minutes. The assays are started by the addition of 5-50 μl of 5-alpha-reductase (rat or human) in the presence or absence of inhibitors (SPB-extract from different sources and at different concentrations). Proscar™ (finasteride) is used as a positive control. The reaction is stopped by the addition of 1 ml diethylether containing 25 μg each of T, DHT, and 3-alpha-androstanediol. Each tube is vortexed, centrifuged, and the top ether layer separated. The ether is evaporated with a stream of nitrogen and the residue dissolved in 20 μl chloroform/methanol (2:1, v/v) and applied to thin layer plates (silica gel 60, E. Merck, 5748-7, Darmstadt, Germany). The plates are developed with chloroform/ethyl acetate (3:1, v/v), autoradiographed at -70°C for 18 hours and radioactive zones (corresponding to T, DHT, and 3-alpha-androstanediol) cut and counted for radioactivity in a liquid scintillation counter. The method is used to calculate K_m and V_{max} values and percent inhibition of the conversion of T to DHT in presence of a SPB-extract at different concentrations.

6.6. CHEMICAL ANALYSIS GC-MS, HPLC OF OLEIC, LAURIC, LINOLEIC, LINOLENIC, PALMITIC, MYRISTIC ACIDS BOTH FREE FATTY ACIDS AND ETHYL ESTERS

Saw Palmetto: methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, ethyl laurate, methyl linolenate, ethyl myristate, methyl caproate, ethyl palmitate, methyl caprylate, methyl caprate, ethyl oleate, methyl laurate, ethyl linolenate, methyl myristate and ethyl linolineate.

Saw Palmetto:	% w/w				
	C71548#1	C71548#2	C71548#3	C71548#4	C71548#5
methyl palmitate	0.004	0	0.276	0.019	0.304
methyl stearate	0.249	0	0.728	0.049	0.714
methyl oleate	0.462	0.058	0.875	0.078	1.069
methyl linoleate	5.418	0.67	9.911	0.956	13.401
methyl linolenate	2.184	0.285	4.243	0.453	5.448
methyl caproate	2.225	1.14	3.24	0.552	3.059
methyl caprylate	0.332	0.297	0.57	0.59	0.608
methyl caprate	0	3.276	13.871	1.102	11.67
methyl laurate	1.805	7.291	3.527	0.249	2.916
methyl myristate	0.121	0.815	0.191	0.011	0.269
ethyl laurate	0	0	0.036	0	0.004
ethyl myristate	0	0	0.019	0.002	0.004
ethyl palmitate	0.23	0.001	0.017	0.004	0.138
ethyl oleate	0	0	0.069	0.002	0
ethyl linoleate	0.002	0	0.115	0.01	0
ethyl linolineate	0	0	0.002	0	0.003
Totals:	13.028	13.833	37.414	4.058	39.303

6.7. THE ESTABLISHMENT OF THE PHARMAPRINT

Five commercially available extracts of Saw Palmetto were quantitatively analyzed to determine the amount of several fatty acids and fatty acid esters present in each extract. The results are depicted in FIG. 4. The figure

shows the wide variability of each of the components present in the various extracts.

Each of the fatty acids and fatty acid esters, *i.e.*, the components, of FIG. 4 were analyzed to determine their activity in four bioassays relevant for the BPH clinical indication, see above. None of the components showed acitvity against Cox-2. The activities, IC_{50}s, for purified components in the other three bioassays were as follows: linolenic acid (233 μM in COX-1, 12 μM in 5-LIPO); linoleic acid ethyl ester (6 μM in androgen receptor assay); lauric acid ethyl ester (130 nM in androgen receptor assay); and β-sitosterol (~10μM in the androgen receptor assay). Because none of the extracts were active in the COX-1 and 5-LIPO assay, the androgen receptor assay was selected for the calcualtions shown below.

The contribution of each individual component to the observed total bioactivity is calculated using (i) the total bioactivity of the botanical extract, (ii) the amount of each component present in each extract and (iii) the IC_{50}s of each purified component. This calculation is exemplified below using commercial sample #3 and the androgen receptor assay. Sample #3 has a total extract IC_{50} value for the androgen receptor of 3.5 μM (total bioactivity), assuming the average molecular weight of the components is 200. A capsule of sample #3 contains the following proportions of the ethyl esters of lauric acid (0.036 W/W%; 228 MWt) and linoleic acid (0.115 W/W%; 308 MWt).[1] A calculation of the per cent contribution of the androgen receptor bioactivity of lauric acid ethyl ester relative to the total extract bioactivity is made using the following formula: the extract IC_{50} bioactivity (3.5μM = 3,500nM; average MWt. <200>) is multiplied times the amount of lauric acid ethyl ester present (0.036%W/W) and then divided by the lauric acid ethyl ester observed IC_{50} (130

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[1] β-sitosterol is present in ~0.2% W/W of the total extract. The activity of purified β-sitosterol (~10 μM). Due to the preliminary nature of these results, β-sitosterol is not included in the calculation.

nM) and multiplied by 100 and corrected for the molecular weight (3,500nM x 200 MWt x 0.0355 x 100)/(130nM x 228 MWt) = 83.9%]. The per cent contribution of linoleic acid ethyl ester using the same formula is calculated as follows: (3.5μM x 200 x 0.1146 x 100)/(6μM x 308) = 4.4%. Thus, two of the components in combination, lauric acid ethyl ester and linoleic ethyl ester, account for 90% of the observed *in vitro* bioactivity in this assay and are defined as active components in the androgen receptor bioassay.

The combined bioactivity of the lauric and linoleic ethyl esters is used to define a bioactivity standard for acceptance or rejection of pharmaceutical grade compositions.[2]

The bioactivity ranges are set to determine if a given botanical qualifies as a pharmaceutical grade botanical as follows: In one calculation, the requirements are set such that the active components must account for 25% of the bioactivity based on the active component standard described above. Given the bioactivity required (25% of the standard) and known bioactivity for the active component, the calculation is as follows: The weight percent of the active component multiplied by the minimal percentage of the biological activity required, e.g., (0.036% W/W x 25% = 0.009% W/W) for lauric acid. Similarly, for linoleic acid must account for 0.42%. Alternatively, the requirements are established such the combination of the two esters accounts for at least 25% of the observed bioactivity.

Requiring that each component account for 50% of the bioactivity, the sample must contain at least 0.018% W/W lauric acid ester or 0.84% linoleic acid ester.

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[2] Extract #2 displayed inconsistent behavior based on analysis in the chemical and biological assays. Extract #2 has apparent androgen receptor assay activity four times stronger than the best standard tested (30nM *vs.* 130 mN for lauric acid ethyl ester). However, extract #2 contains very low amounts of putative components. Thus, extract #2 is not part of the analysis.

Requiring that each component account for 70% of the bioactivity, the sample must contain at least 0.025% W/W lauric acid ester or 1.176% W/W linoleic acid ester.

Requiring that each component account for 80% of the bioactivity, the sample must contain at least 0.029% W/W lauric acid ester or 1.344% linoleic acid ester.

Using either combined lauric and linoleic ethyl esters or the individual esters bioactivities, we can set now clearly defined standards for acceptance or rejection for pharmaceutical grade compositions of each of the five commercial samples tested on the basis of % W/W of these esters. For the five commercial samples shown in FIG. 4, even using the least rigorous requirements, e.g. 25%, samples 1, 2, 4 and 5 are rejected as unsuitable for pharmaceutical grade drug compositions due to the levels of the two esters determined for each sample.

7. EXAMPLE: KOREAN MISTLETOE

7.1. PLANT SOURCE/EXTRACTION METHOD

Plant powder (2.4 Kg) obtained from Korean mistletoe was extracted with 300 ml batches of water in a clean blender. The extract was filtered through cheese cloth-lined filter beds to eliminate fibrous and water-insoluble residues, final volume 6.03 liters. Final concentration of the extract was 39.8% (plant weight/volume).

7.2. FRACTIONAL ANALYSIS

The extract was left at 4°C for two weeks in absence of air (flushed with nitrogen). At this time, additional insoluble residues were deposited. The cold extract was filtered through 0.8μ filters and final sterile filtration was performed with 0.2 μ filters in a sterile environment. The semipurified product was collected in 500 ml sterile vacuum containers and identified as T4GEN. The product samples were found to be pyrogen free. Samples from the flask were removed with a sterile syringe in a laminar flow

hood and diluted on the basis that each ml of the sample contains 400 mg of the extract (40% solution).

The extract samples were analyzed according to the affinity chromatography system schematically shown in FIG. 9, which was the same system used to establish the standard marker fingerprints. The columns used to separate the proteins were Sepharose™ 4B.

The columns were prepared as follows:

Activation of Sepharose™ 4B: Sepharose™ 4B (400 ml) was repeatedly washed with double distilled water and filtered on a buchner funnel. The sepharose residue was repeatedly washed with Na_2CO_3 (0.5 M, pH 11) and then suspended in a stirred 2 liter cylinder in 400 ml Na_2CO_3 (0.5 M, pH 11). The cylinder was covered with aluminum foil and to the stirred suspension of Sepharose™ 4B, divinylsulfone (48 ml, absence of light) was added dropwise over a period of 80 minutes. The reactants were stirred for another 30 minutes at room temperature. Then the resin was filtered on a sintered glass funnel (no touching with hands or paper) with approximately 500 ml of Na_2CO_3 (0.5 M, pH 10, make with $NaHCO_3$). At this time the resin was suspended in 400 ml Na_2CO_3 (0.5 M, pH 10) and used for preparing sugar-specific affinity resins as follows:

Galactose-specific Sepharose™ 4B: To the activated resin 380 ml (in 5M Na_2CO_3), galactose (38 g) was added with stirring in absence of light. The suspension was stirred overnight and then the suspension was filtered on a sintered glass funnel. To inactivate the reacted activated sepharose, the residue was washed with 0.5 M $NaHCO_3$ (pH 8.5) and then suspended in 350 ml $NaHCO_3$ (0.5 M, pH 8.5) and 14 ml 2-mercaptoethanol. The stirred suspension was maintained at room temperature for 4 hours and then filtered on a sintered glass funnel. The resin was washed with 0.2 M PBS (phosphate buffered saline) and finally suspended in 380 ml 0.2 M PBS and stored at 4°C along with a few crystals of NaN_3.

Lactose-specific Sepharose™ 4B: The method of preparation was the same as described for galactose. Here

300 ml of activated Sepharose™ was reacted with 30 g of lactose and the affinity resin was deactivated with 300 ml $NaHCO_3$ (0.5 M, pH 8.5), 12 ml 2-mercaptoethanol and finally suspended in 300 ml of PBS and NaN_3 as described in the previous preparation.

N-acetyl-D-galactosamine-specific Sepharose B: Activated Sepharose 4B (30 ml) was treated with 3 g of N-acetyl-D-galactosamine as described. The reaction was terminated with 30 ml $NaHCO_3$ (0.5 M, pH 8.5) and 5 ml 2-mercaptoethanol. The resin was maintained in 30 ml PBS and a few crystals of NaN_3 at 4°C.

Fucose-specific-Sepharose™ 4B: Activated Sepharose™ 4B (50 ml) was reacted with 5 g fucose. The affinity resin was treated with 50 ml $NaHCO_3$ and 5 ml 2-mercaptoethanol to deactivate the unreacted Sepharose™. The resin was maintained in 50 ml PBS and NaN_3 as described.

Melibiose-Specific Sepharose™ 4B: Activated Sepharose 4B (50 ml) was reacted with 5 g melibiose. The reaction was terminated with 50 ml $NaHCO_3$ and 5 ml 2-mercaptoethanol. The resin was maintained in 50 ml PBS and a few crystals of NaN_3 at 4°C.

The same methods can be used to provide columns of different sugar specificity. The used columns were regenerated by elutions with 5 M urea and followed by elution with 0.5 M $NaHCO_3$ (pH 8.5). Prior to use, columns are equilibrated with 0.02 M Tris/HCl buffer (Buffer C).

The Buffers used for Extraction and Affinity Chromatography were prepared as follows:

All buffers made in double distilled water (DD).

A) Tris/HCl (0.02 M, pH 7.8) containing NaCl (0.2 M) dithiothreitol (1 mM) and just prior to use add phenyl methanesulfonyl fluoride (0.01 mM). (Buffer A).

B) Tris/HCl (0.02 M, pH 7.8) containing 0.4 M KCl, 2% Triton x-100, 1 mM dithiothreitol and 0.01 mM phenyl methanesulfonyl fluoride (to be added before use). (Buffer B).

C) Tris/HCl (0.02 M, pH 7.8) containing 1.25 M NaCl, 25 mM $CaCl_2$, 0.05% Triton x-100 and 1 mM dithiothreitol. (Buffer C).

D) Buffer (C) containing 4 mM EDTA instead of 25 mM $CaCl_2$. (Buffer D).

If desired, EGTA may be substituted for EDTA.

A known volume of the mistletoe extract was adjusted to pH 7.8 with 2 M Tris-buffer. The solution was absorbed on a series of Sepharose™ 4B affinity columns (1.6 x 7 ml). The columns were washed with excess (200 ml) of 0.02 M Tris-buffer (pH 7.8) containing 25 mM $CaCl_2$ (Buffer C) to remove all unbound proteins (viscotoxins and alkaloids). Then each column was separately washed with Tris-buffer (pH 7.8) containing 4 mM EDTA (Buffer D) to elute proteins which require Ca^{++} for their binding to specific sugars *i.e.* Ca^{++} dependent sugar-binding proteins (100 ml samples). Subsequently, the columns were washed with Tris-buffer (Buffer C, 200 ml) and then eluted with the same buffer (100 ml) containing 0.5 M corresponding sugars to remove non-Ca^{++} dependent sugar binding proteins. The unbound proteins were fractionated on a Sephadex™ G-75 column (2.5 x 75 cm) to separate viscotoxins from alkaloids. All fractions were dialyzed to remove salts and other buffer ingredients. Each dialyzed fraction was concentrated by an Amicon™ concentrator using DIAFLO™ ultrafiltration membrane YM10 (10,000 cutoff). Protein concentration was measured by Bio-Rad assay with bovine-globulin as a standard (each separated protein may be characterized for its purity and molecular weight by SDS page gel chromatography).

7.3. BIOLOGICAL ACTIVITY ANALYSIS

The inhibitory concentration (IC_{50}) was determined for each sugar-binding protein as follows:

Leukemia L1210 cells were maintained in asynchronous logarithmic growth at 37°C in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine calf serum and 1 % (v/v) Pen

Strep. The cell population doubling time was 11-12 hours. The cells were passed every 48 hours at 1 x 10^4 cells/ml in order to keep the cells in a logarithmic stage of growth.

For all growth inhibition studies all stock solutions and dilutions were made with sterile 0.7% NaCl solution. The cell cultures were seeded at 2-5 x 10^4 cells/ml in duplicates for each inhibitor concentration in a microtiter plate (0.18 ml/well). The covered microtiter plate was incubated for 48 hours in a humidified CO_2 incubator containing 5% CO_2 in air. At the end of the incubation period, aliquots of each well were added to a measured volume of isotonic saline and counted in an electronic counter. Because fractions at high concentrations caused rapid cellular fragmentation, the test microtiter-plates were routinely checked under a microscope prior to cell number counting so that the results were not compromised. Cell viability was determined by trypan blue exclusion. The results were calculated by plotting percent cell growth inhibition (as compared to the cell density of the saline treated controls) versus log of protein (or specific fraction) concentration which caused 50% inhibition (IC_{50}) in cell growth as determined from the graph.

The results of the analysis are shown in Tables 10 and 11 for n-T4GEN salt and detergent extracts prepared in accordance with this example. As can be seen from Tables 10 and 11, the extracts from both the sap and the cell walls of the Korean mistletoe have protein levels and inhibitory activities which all fall within the limits required to be identified as pharmaceutical grade extracts in accordance with the present invention. Accordingly, these extracts may be used in clinical studies directed to cancer or AIDS treatment. They may also be used for routine patient treatment since their quality and efficacy have been established in accordance with the protein fingerprint identifiers as required by the present invention.

TABLE 10

FRACTIONATION OF THE VARIOUS CONSTITUENTS OF V. ALBUM CORATUM WITH ANTILEUKEMIA - L1210 ACTIVITY - n-T4GEN (40 PERCENT EXTRACT)

(Affinity Method, Fractionation of Salt Extract)

Fraction identity	Protein Content (mg/ml)	Total Volume (ml)	Total Protein (mg)	IC^{a}_{50} (μg Protein/ml)	Total Activity[b] Units
Salt Extract	7.63	350	2673	0.11	2.4×10^{7}
Affinity columns eluted with EDTA buffer - Ca^{++} dependent					
1. Lactose	0.31	20	6.24	0.38	1.6×10^{4}
2. Galactose	0.39	20	7.91	0.25	3.1×10^{4}
3. Melibiose	0.28	20	5.44	0.20	2.7×10^{4}
4. N-Acetyl-D-galactosamine	0.70	12.5	875	0.29	3.00×10^{4}
5. Fucose	0.28	18	5.04	0.36	1.4×10^{4}
Affinity columns eluted with corresponding sugars - non-Ca^{++} dependent					
1. Lactose	0.27	22	5.98	0.00027	2.2×10^{7}
2. Galactose	1.40	9	12.60	0.0013	9.6×10^{6}
3. Melibiose	0.32	10	3.20	0.0034	9.4×10^{5}
4. N-Acetyl-D-galactosamine	0.66	18	11.90	0.017	7.0×10^{5}
5. Fucose	1.17	15	2.59	0.019	1.3×10^{5}
Sephadex - G75 (Unbound proteins) Fractions[c]					
I (12-50)	1.84	46	84.6	0.5	1.69×10^{5}
II (51-70)	1.22	22.5	27.45	4.0	6.8×10^{3}
III (71-100)	1.12	40	44.80	2.8	1.6×10^{4}
IV (101-140)[d]	-0-	50	-0-	13.5	1.6×10^{4}

[a] Inhibitory concentration expressed as μg protein/ml which caused 50% inhibition of the growth of L1210 cells in culture.

[b] Activity unit is defined as dilution factor needed for a specific fraction which when added to L1210 cells caused a 50% cell growth inhibition.

[c] 50 ml on column from a total eluate of 325 ml.

[d] 31 mg alkaloids obtained from fraction IV.

TABLE 11

FRACTIONATION OF THE VARIOUS CONSTITUENTS OF V. ALBUM CORATUM WITH ANTILEUKEMIA - L1210 ACTIVITY (40 PERCENT EXTRACT)

(Affinity Method, Fractionation of Detergent Extract)

Fraction identity	Protein Content (mg/ml)	Total Volume (ml)	Total Protein (mg)	IC^{a}_{50} (μg Protein/ml)	Total Activity[b] Units
Detergent Extract	1.68	450	756	0.27	2.8×10^{6}
Affinity columns eluted with EDTA buffer					
1. Lactose	0.18	22	4.04	0.250	1.6×10^{4}
2. Galactose	0.08	11.5	0.97	0.031	3.1×10^{4}
3. Melibiose	0.16	20	3.12	0.054	5.7×10^{4}
4. N-Acetyl-D-galactosamine	0.17	19	3.20	0.076	4.2×10^{4}
5. Fucose	0.12	6.5	0.78	0.100	0.5×10^{4}
Affinity columns eluted with corresponding sugars					
1. Lactose	0.35	95	3.23	0.0045	2.1×10^{6}
2. Galactose	0.50	15	7.50	0.0055	1.3×10^{6}
3. Melibiose	0.15	12	0.61	0.0084	0.07×10^{6}
4. N-Acetyl-D-galactosamine	0.62	4	7.68	0.0035	2.1×10^{6}
5. Fucose	0.50	10	5.10	0.0500	0.1×10^{6}
Sephadex - G75 (Unbound proteins) Fractions[c]					
I (9-35)	1.58	33	52.1	1.25	0.05×10^{6}
II (36-55)	1.36	20	27.2	1.70	0.008×10^{6}
III (56-120[d])	-0-	50	-0-	14.00	0.002×10^{6}

[a] Inhibitory concentration expressed as μg protein/ml which caused 50% inhibition of the growth of L1210 cells in culture.

[b] Activity unit is defined as dilution factor needed for a specific fraction which when added to L1210 cells caused a 50% inhibition.

[c] 50 ml on column from a total eluate of 420 ml.

[d] 22 mg alkaloids obtained from fraction III.

The protein containing fractions (12-100) with biological activity in L1210 system obtained from Sephadex™ G75 columns (see Table 10) contained a mixture of viscotoxins (1.0 1 9 g). The fractions (101-140) were combined and extracted with 3 x 200 ml chloroform. The chloroform layer was dried over anhydrous Na_2SO_4 filters and the filtrate was evaporated under vacuum to obtain 201 mg of alkaloids (weights of viscotoxins and alkaloids set forth in Table 10 are adjusted to a total of 325 ml for the unbound fraction obtained from affinity columns). Thus 1 ml of the 1 percent extract contained 4.9 pg lectins; 72 pg unbound proteins which contains from 10 to 40 pg of viscotoxins; and 14.3 μg alkaloids.

7.4. PREPARATION OF PHARMACEUTICAL GRADE WATER EXTRACT

An extract from Korean mistletoe was prepared by taking a known weight of plant (100 g) and cleaning and crushing it in the presence of double distilled water to form a 40% by weight solution of mistletoe. It is preferred that the plant be cut and the cuttings put in a plastic bag and flash frozen in liquid nitrogen prior to being crushed and combined with the distilled water. The crushing and combination of the frozen material with the distilled water is preferably carried out in a blender for about 2 minutes. The resulting mixture is centrifuged at 10,000 rpm for 65 minutes to separate extract from insoluble residue. The residue is twice extracted with known volumes (100 ml) of water to remove all extract and subjected to centrifugation. The supernatants are combined. The resulting extract is stored in the absence of air at room temperature for two weeks. The extract is then sterilized by step filtration as is conventionally known.

The sterile extract is then subjected to a preliminary screening test as previously described to determine if its bioactivity with respect to the L1210 leukemia system is 100 A.U. or more. If the extract passes this test, then it is subjected to the more rigorous tests as described in the

prior examples to determine its sugar-binding protein fingerprint. If the protein levels fall within the limits required in accordance with the present invention, then the extract is identified as pharmaceutical grade. Table 11 sets forth the results of testing of an extract prepared as above. As can be seen, the extract has concentration levels and bioactivity values which fall within the standard fingerprint limits which are required for it to be identified as pharmaceutical grade in accordance with the present invention.

TABLE 12

FRACTIONATION OF DIFFERENT BIOLOGICALY ACTIVE CARBOHYDRATE BINDING PROTEINS FROM *VISCUM ALBUM C.* EXTRACT (40%, FrF) (n-T4GEN)

(Affinity Method, Fractionation of Detergent Extract)

Fraction identity	Total Volume (mg/ml)	Total Protein (ml)	IC_{50}[a]	IC_{50}[a] (μg/ml)	Total Activity Units[b]
Extract (40%, FrF)	90	234	32,500		2.92×10^6
Affinity columns eluted with corresponding sugars					
1. Lactose	8	2.24	208,000	0.001	1.66×10^6
2. Galactose	11	2.68	68,000	0.0035	0.75×10^6
3. Melibiose	6.5	0.96	5,600	0.025	0.036×10^6
4. N-Acetyl-D-galactosamine	4.5	0.81	49,000	0.0035	0.22×10^6
5. Fucose	5	0.33	10,500	0.0056	0.052×10^6
Sephadex - G75 (Unbound proteins) Fractions[c]					
I (9-35)	1.58	33	52.1	1.25	0.05×10^6
II (36-55)	1.36	20	27.2	1.70	0.008×10^6
III (56-120[d])	-0-	50	-0-	14.00	0.002×10^6
Unbound proteins and alkaloids	168	285	1,600	1.25	0.27×10^6

[a] Dilution factor needed per ml to cause 50% inhibition of L1210 cells in culture.
[b] Activity unit is defined as dilution factor needed for a specific fraction which when added to L1210 cell will cause a 50% inhibition of the cell growth.

7.5. PREPARATION OF PHARMACEUTICAL GRADE TOTAL EXTRACT OF KOREAN MISTLETOE

An extract of Korean mistletoe was prepared by taking 100 g of mistletoe, freezing it, and powdering it. The frozen powder was then thawed and extracted with 200 ml of cold acetone. The extraction mixture was centrifuged at 10,000 rpm for 60 minutes. The precipitate was washed with two 100 ml aliquots of cold acetone and centrifuged again at 10,000 rpm for 60 minutes. The resulting extract residue is extracted with two 200 ml aliquots of Buffer A (see Example 8) in a blender for 2 minutes each. The two extract aliquots are centrifuged at 10,000 rpm for 60 minutes and the supernatants combined. The extract residue is extracted a final time with an additional 200 ml of Buffer A. After centrifugation, this final extractant is combined with the other two aliquots to form a salt extract which, after removal of salts, is then tested in accordance with the present invention to determine if its sugar-binding protein fingerprint meets the pharmaceutical grade requirements set forth above.

The extract residue remaining after the above extraction with Buffer A may be extracted with a detergent extract - Buffer B (see Example 8)- to form an extract which, after dialysis to remove detergents and salts, also can be tested to determine if it meets the pharmaceutical grade requirements of the present invention.

8. EXAMPLE: EUROPEAN MISTLETOE

An extract of European mistletoe (*Viscum album L.*) was prepared and analyzed according to Example 7 except that only the aqueous salt extract was analyzed for its sugar-binding protein fingerprint. The extract is identified as T4GEN. The results of the fingerprint determination are set forth in Table 12. The T4GEN extract meets the requirement of the established standard fingerprint and therefore qualifies as a pharmaceutical grade extract.

Table 13 sets forth the analysis of ISCADOR™ which is a fermented mistletoe extract. This fraction meets some, but not all, of the requirements of the standard fingerprint. For example, the bioactivity of the melibiose and fucose fractions are too low.

Table 14 shows the results of bioactivity assays for the T4GEN and ISCADOR™ extracts where the IC_{50} is expressed as the dilution factor needed per ml to cause 50% inhibition of L1210 cells in culture. Table 14 is derived from Tables 12 and 13 and shows the general drop in ISCADOR™ activity compared to the unfermented European extract (T4GEN).

TABLE 13

FRACTIONATION OF BIOLOGICALLY ACTIVE CARBOHYDRATE BINDING PROTEINS FROM T-4GEN

(Affinity Method, Fractionation of Salt Extract)

Fraction identity	Protein Content (mg/ml)	Total Volume (ml)	Total Protein (mg)	IC^{a}_{50} (μg Protein/ml)	Total Activity[b] Units
T4GEN (10%, FrF)	0.9	100	90	0.32	4.6×10^5
Affinity columns eluted with EDTA buffer (Ca^{++} dependent sugar-binding proteins)					
1. Lactose	0.236	7	1.652	0.0337	0.49×10^5
2. Galactose	0.040	9	0.360	0.0035	1.00×10^5
3. Melibiose	0.052	4	0.208	0.0042	0.44×10^5
4. N-Acetyl-D-galactosamine	0.128	8	1.024	0.080	0.12×10^5
5. Fucose	0.920	8	0.734	0.050	0.14×10^5
Affinity columns eluted with buffer containing corresponding sugars (Non-Ca^{++} dependent sugar-binding proteins)					
1. Lactose	0.158	11	1.738	0.0079	2.2×10^5
2. Galactose	0.128	10	1.280	0.0156	0.8×10^5
3. Melibiose	0.200	8	1.600	0.345	0.4×10^5
4. N-Acetyl-D-galactosamine	0.140	10	1.140	0.0044	3.2×10^5
5. Fucose	0.148	9	1.332	0.360	0.36×10^5

[a] Inhibitory concentration expressed as μg protein/ml which caused 50% inhibition of the growth of L1210 cells in culture.

[b] Activity unit is defined as dilution factor needed for a specific fraction which when added to L1210 cells caused a 50% cell growth inhibition.

TABLE 14

FRACTIONATION OF BIOLOGICALLY ACTIVE CARBOHYDRATE BINDING PROTEINS FROM ISCADOR 'M', (20%)

Fraction identity	Protein Content (mg/ml)	Total Volume (ml)	Total Protein (mg)	IC'_{50} (μg Protein/ml)	Total Activity[b] Units
Iscador 'M', 20%	4.6	12	55	0.25	2.2×10^5
Fractions from G-75 column (21-65)	0.315	65	20.50	0.078	2.6×10^5
Affinity columns eluted with EDTA buffer					
1. Lactose	0.203	10	2.08	0.039	0.57×10^4
2. Galactose	0.064	12	0.70	0.07	0.63×10^4
3. Melibiose	0.182	12	2.20	0.90	1.20×10^4
4. N-Acetyl-D-galactosamine	0.048	12	0.50	0.29	0.35×10^4
5. Fucose	0.151	9	1.36	0.28	0.80×10^4
Affinity columns eluted with buffer containing corresponding sugars					
1. Lactose	0.053	8.5	0.45	0.30	0.15×10^4
2. Galactose	0.080	9	0.70	0.26	0.27×10^4
3. Melibiose	0.009	7	0.06	0.035	0.17×10^4
4. N-Acetyl-D-galactosamine	0.066	12.5	0.82	0.027	3.00×10^4
5. Fucose	0.198	10.5	2.07	0.55	0.38×10^4
Unbound proteins (after sugar buffer elution)	0.225	50	11.25	0.25	4.5×10^4

[a] Inhibitory concentration expressed as μg protein/ml which caused 50% inhibition of the growth of L1210 cells in culture.

[b] Activity unit is defined as dilution factor needed for a specific fraction which when added to L1210 cells caused a 50% cell growth inhibition.

TABLE 15

EFFECT OF FERMENTATION ON THE BIOLOGICAL ACTIVITY OF CARBOHYDRATE BINDING PROTEINS (LECTINS) OF VISCUM ALBUM L. (ISCADOR™ 'M')

Protein	T4GEN (A.U.)	ISCADOR™ (A.U.)	% Change (±)
(Ca^{++}) dependent sugar-binding proteins			
1. Lactose	0.49×10^5	0.02×10^5	-95.0
2. Galactose	1.00×10^5	0.12×10^5	-88.0
3. Melibiose	0.44×10^5	0.07×10^5	-84.1
4. N-Acetyl-D-galactosamine	0.12×10^5	0.01×10^5	-97.1
5. Fucose	0.12×10^5	0.02×10^5	-85.7
(Non-Ca^{++} dependent sugar-binding proteins)			
1. Lactose	2.20×10^5	0.20×10^5	-91.0
2. Galactose	0.80×10^5	0.02×10^5	-97.5
3. Melibiose	0.40×10^5	0.06×10^5	-85.0
4. N-Acetyl-D-galactosamine	3.20×10^5	0.72×10^5	-77.5
5. Fucose	0.36×10^5	0.01×10^5	-97.3

ISCADOR™ 'M', 20% and T4GEN (10%) were used as examples of fermented and unfermented mistletoe clinical preparations. Activity is described as total activity units as described elsewhere.

9. EXAMPLE: ST. JOHN'S Wort, *Hypericum perforatum*

9.1. PLANT SOURCE/EXTRACTION METHOD:

There are many sources and many forms of St. John's Wort. It may be dried (stem, leaf, flowers, buds). It may also be in the form of a powdered extract. It may be freeze-dried. An oil extract of the crushed flowers may be prepared. Various forms of infusions (aqueous), oil macerates, or alcohol water extractions are available. The methods of processing and extracting St. John's Wort are discussed in the detailed description of the invention above.

9.2. COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are many commercial suppliers of St. John's Wort and extracts of St. John's Wort, including the following listing: Jarsin™, Jarsin™ 300 (LI160) (Lichtwer Pharma GmbH, Berlin), Psychotonin™ M, Psychotonin™ forte (Hersteller,

Darmstadt), Hyperforat™, Extract™ Z 90017, Neuroplant™, Neuropas™, Esbericum™, Remotiv™ (Bayer, Germany) and Sedaristan™. The following companies also produce St. John's Wort commercially: PhytoPharmica, Nature's Way, Herbal Choice, Botalia Gold and Herb Pharm.

There is one commercially available hypericin product called VIMRxyn™ available from VIMRx Pharmaceuticals™.

9.3 CLINICAL STUDIES

St. John's Wort has been the subject many clinical studies on the extract and the botanical itself. The major clinical indication is the alleviation of mild to moderate depression. Other clinical indications include AIDS, antibacterial uses, anticancer uses, antimutagenic uses, antiviral uses, use as an immunostimulant and use for immunosuppression. These will be discussed in greater detail in the sections that follow.

9.3.1. MILD-TO-MODERATE DEPRESSION

St. John's Wort has become increasingly popular in Germany where physicians routinely prescribe herbal medicines. In 1994, 66 million daily doses of St. John's Wort were prescribed there for use in the treatment of depression (De Smet and Nolen, 1996, *British Medical Journal* 313:241-247). This phytomedicine has now been tested in more than 3,000 patients against placebo and various active medications (Hänsgen et al., 1994, *Nervenheilkunde* 12:285-289; Harrer et al., 1994, *J. Geriatric Psychiatry Neurology* 7:S24-28; Hübner et al., 1994, *J. Geriatric Psychiatry Neurology* 7:S12-14; Martinez et al., 1994, *J. Geriatric Psychiatry Neurology* 7:S29-33; Sommer and Harrer, 1994, *J. Geriatric Psychiatry Neurology* 7:S9-11; Vorbach et al., 1994, *J. Geriatric Psychiatry Neurology* 7:S19-23; Woelk et al., 1994, *J. Geriatric Psychiatry Neurology* 7:S34-38). The primary formulations used in the German clinical trials are summarized in Table 15. All these formulations are standardized according to hypericin content.

TABLE 15:
Description of the Primary
St. John's Wort Preparations Used in the Depression Studies

Preparation name	Total Extract/day	Total Hypericin/day
Jarsin™ (tablets)	900 mg	1 mg
Jarsin™ 300 (tablets)	900 mg	2.7 mg
Psychotonin™ (drops)	350-500 mg	0.5-0.75 mg
Neuroplant™ (caps)	500 mg	1 mg
Hyperforat™	N/A	0.4-0.6 mg
Sedariston™*	300-600 mg	0.45-0.9 mg

*Also contains valerian extract

German researchers (with a colleague from San Antonio, Texas) recently published a meta-analysis of 23 randomized trials of St. John's Wort with a total of 1,757 outpatients with mild to moderately severe depressive disorders. They concluded that the herb was significantly superior to placebo, and appeared comparably effective to standard antidepressants while producing fewer side effects (Linde, 1996, *British Medical Journal* 313:253-258).

The controlled studies of St. John's Wort's efficacy in depression included in the meta-analysis were randomized, or "quasi-randomized" through alternation. Comparisons were made of the herb alone, or in combination with other plant extracts, to placebo and/or a standard antidepressant. Twenty of the 23 trials were double-blind, one was single blind, and two were open label. Most were 4 to 8 weeks in duration. The methodological quality of each study was assessed by at least two reviewers to determine eligibility for inclusion in the meta-analysis.

In each study, improvement in depressive symptoms had been evaluated with depression scales with interrater reliability, most commonly the Hamilton depression scale (HAM-D) and the clinical global impressions index (CGI). The

daily dose of either hypericin, the reference substance for standardization, or of total extract varied considerably between studies, from 0.4, 2.7 mg and 300 and 1000 mg, respectively.

In 13 studies comparing a single St. John's Wort preparation with placebo, 55.1 percent (225) patients receiving the herb were improved, compared with 22.3 percent (94) responding to the placebo. In the comparisons to standard antidepressants, in three trials with single preparations and two with combinations, 63.9 percent (101) patients responded to single preparations compared with 58.5 percent (93) with standard antidepressants, and 67.7 percent (88) responded to combination extract products (St. John's Wort and *Valeriana*) compared with 50 percent (66) with standard antidepressants.

The researchers acknowledged the problems in drawing valid conclusions from the pooled data of quite heterogeneous studies. These problems are compounded by the different amounts and preparations of the herb used across the studies and the possibility that hypericin is not the only bioactive component.

These limitations aside, Linde and colleagues find sufficient evidence to conclude that St. John's Wort is better than placebo in treating some depressive disorders. These data were inadequate, however, to judge whether it is as effective as standard antidepressants, although it appears to cause fewer side effects. They consider that these initial indications of efficacy warrant the undertaking of longer controlled trials comparing several doses of different St. John's Wort preparations to standard antidepressants.

In separate commentary accompanying the meta-analysis, Netherlands researchers Peter De Smet and Willen Nolen agreed that these data are promising, but not yet sufficient to accept St. John's Wort as an effective antidepressant preparation (De Smet and Nolen, 1996, *British Medical Journal* 313:241-247). Besides the need for dose standardization and adequate trial lengths, they call for studies in severely

depressed patients and long-term studies to assess the risk of relapse and emergence of late side effects (De Smet and Nolen, *supra*).

In another single blind study of patients with Seasonal Affective Disorder (DSM-III-R criteria) it was observed that 900 mg of St. John's Wort daily was equal to the effects of conventional light therapy (Kapser et al., 1996, 2nd International Congress on Phytomedicine, Munich).

Animal Studies

A commercial standardized extract of St. John's Wort (Psychotonin™) was tested in several animal models predictive of psychotropic activity (Okpanyi and Weicher, 1987, *Arzneim-Forsch* 37:10-13). These activities included two used for antidepressants: increased activity in a water wheel test in mice, and reduced aggressiveness in isolated male mice.

In a recent presentation, Butterweck et al., compared the St. John's Wort extract, LI 160, with bupropion, a synthetic antidepressant (Butterweck et al. 2nd International Congress on Phytomedicine. Munich; 1996). The authors found that both drugs resulted in similar effects including the tail suspension test (mice) and the forced swim test (rats). Since St. John's Wort treatment was antagonized by drugs known to reduce dopamine functional activity (haloperidol, sulpiride, χ-methyltyrosine, and γ-butyrolactone), the authors concluded that St. John's Wort exerts its activity via dopaminergic activation. Studies on various fractions of the methanol extract revealed that different constituents were responsible for the various activities.

A hepatoprotective activity of a water/alcohol extract has been reported in mice at a dose of 500 mg/kg intraperitoneally (Öztürk, et al., 1992). This conclusion was based on the ability of St. John's Wort to increase bile duct flow in rats and to reduce CCl_4-induced narcosis in barbiturate treated mice. Müller et al., (1996) reported that the St. John's Wort extract, LI 160, resulted in a 15% down-regulation of β-adrenergic receptors in the rat frontal

cortex after subchronic treatment (250 mg/kg for 2 weeks). In the same study, these authors found a 25% down-regulation after similar doses (10 times the clinical dose) of imipramine.

9.3.2. ANTIVIRAL ACTIVITY

Hypericin is currently in early clinical trials in the U.S. as an antiviral (Bombardelli and Morazzoni, 1995; Meruelo et al., 1988). Studies have shown that two of St. John's Wort's primary components, hypericin and pseudohypericin, inhibit a variety of encapsulated viruses, including herpes simplex (Weber et al., 1994) and the human immunodeficiency virus type 1 (HIV-1) virus associated with AIDS (Lavie et al., 1989; Lopez-Bassocchi et al., 1991; Meruelo et al., 1988). While the latter researchers have concluded that hypericin and pseudohypericin display a unique and uncommonly effective antiviral activity, Weber et al., (1994) suggest that it may be due to nonspecific association with cellular and viral membranes. Activity has also been reported against murine cytomegalovirus, Sindbis virus, (Hudson et al, 1991; Lopez-Bazzocchi et al., 1991) and equine infectious anemia virus (Carpenter and Kraus, 1991).

The antiviral activity appears to involve a photoactivation process (Carpenter and Kraus, 1991; Degar et al., 1993; Kraus et al., 1990; Lopez-Bazzocchi et al., 1991) which forms singlet oxygen and inactivates viral fusion and syncytia formation (Lenard et al., 1993; Thomas et al., 1992; Yip et al., 1996). While hypericin does show antiviral activity *in vivo* (mice) these photodynamic properties may limit the potential usefulness as an antiretroviral agent (Stevenson and Lenard, 1993). However, besides singlet oxygen production, hypericin can photo reduce oxygen to superoxide radicals and can form semiquinone radicals in the absence of light (Lavie et al., 1995). These later authors speculate that this ability to form semiquinones might account for the antiviral activity in the whole animals and they have maintained (since the early 1980's) the clinical

utility of hypericin. Hypericin has been reported to inhibit the growth of glioma cells in tissue culture (Couldwell et al., 1994). There appears to be a photoactivation involved in its antineoplastic effect as well (Andreoni et al., 1994).

Hypericin and pseudohypericin have been found to inhibit the important regulatory enzyme, protein kinase C (IC_{50} of 1.7 μg/ml and 15 μg/ml, respectively) (Takahashi et al., 1989). Receptor tyrosine kinase activity of epidermal growth factor is also inhibited by hypericin (de Witte et al., 1993). These later effects have been linked to both the antiviral and antineoplastic activities (Lavie et al., 1995; Panossian et al., 1996).

9.3.3. WOUND-HEALING EFFECTS

St. John's Wort has historically been one of the most relied upon botanicals for the treatment of wounds. Part of this activity is due to St. John's Wort's anti-microbial activity which is attributed to the essential oil. Flavonoids, the phloroglucinol derivatives hyperforin and adhyperforin, and the xanthone kielcorin are also considered to contribute to St. John's Wort's wound-healing effects.

The essential oil and the water soluble fraction of an alcoholic extract exhibit minor antifungal and significant antibacterial activity. The tannins and flavonoids inactivated *E. coli*. At dilutions of 1:400 or 1:200 (Khosa and Bhatia, 1982; Shakirova et al., 1970). Hyperforin and adhyperforin have been reported to possess an antibiotic effect greater than that of sulfonilamide (Negrash and Pochinok, 1972).

A burn ointment prepared by extracting 5 g of fresh flowers with 100 g of olive oil for 10 days at 20°C was used in the treatment of 1st, 2nd and 3rd degree burns. First degree burns heal in 48 hours. Second and third degree burns healed without scarring ≥3 times as rapidly as burns treated with conventional methods. Keloid formation was inhibited (Saljic, 1975). A commercial preparation (Novoimamine; containing 0.412% quercetin) was found to be effective

against *Staphyloccus aureus* infection (Negrash and Pochinok, 1972). In this regard, its effects have similarly been reported to be greater than conventional treatment with sulfonilamide (Aizenman, 1969).

A homeopathic tincture (1:10) of St. John's Wort was studied for its wound-healing properties, and compared with *Calendula officinalis*, another widely used wound-healing herb. The effect of orally administered tincture of St. John's Wort was more pronounced than topical application of *Calendula* tincture in the healing of incision, excision and dead space wounds as evidenced by an increase in epithelization, and wound breaking strength (Gurumadhva et al., 1991). [See Table 16]

TABLE 16
Effects of the Tinctures of St. John's Wort and *Calendula* on Wound-Healing

Wound model		Incision	Excision			Dead space
Parameters studies	Breaking strength (g)	Epithelization period (days)	% wound contraction by day			Granulom (mg)
			5	10	15	
Controls	270.0±22.1	23.00±1.0**	50.0±2.1	70.0±1.2	90.0±0.4	57.0±4.50
St. John's Wort	396.0±18.5	15.00±0.3**	45.0±1.2	88.0±1.0	95.0±1.0	55.3±3.20
Calendula (topical)	354.0±13.5	16.50±1.0**	40.0±2.1	74.0±0.8	99.0±0.5	

n=7, *P<0.001 (Gurumadhva et al., 1991)

9.3.4 MISCELLANEOUS EFFECTS

St. John's Wort has been reported to be useful in a number of additional conditions. In one study the procyanidin (PC) fraction of St. John's Wort was tested in an isolated Guinea pig heart preparation (Melzer, *et al.*, 1989) and found to enhance coronary flow in the same way as the procyanidins from *Crataegus* (Hawthorn). The same researchers tested the procyanidin fractions in porcine isolated coronary

arteries. All PC fractions antagonized histamine or prostaglandin F2 alpha-induced arterial contractions (Melzer, *et al.*, 1991). In another study, preliminary findings suggest that St. John's Wort may be useful in the treatment of chronic tension headaches (Heinze and Göbel, 1996).

9.4. FRACTIONAL ANALYSIS ON A SILICA GEL COLUMN

The fractionation of alcoholic St. John's Wort tincture was performed using normal phase column chromatography. This method was selected as a preferable pre-chromatographic technique on the basis of observed excellent mass recovery (>90%), the separation of the selected standards (flavonoids: mangiferin, rutin, hyperoside, quercitrin, quercitin; hypericin), as well as separation of the other co-ocurring components. A detailed description of the materials and methods utilized is described below.

The chemical markers for St. John's Wort were chosen using the following procedure. A comprehensive search of the literature on St. John's Wort (*Hypericum perforatum*) indicated the hypericins, as well as some of the major flavonoids (rutin, quercetin, quercitrin), as the components with the most consistent bioactivity in a number of assays [flavonoids: analgesic (Vasil'chenko, 1986), sedative (Berghofer, 1987), MAO activity (Kitanov, 1987); hypericin: antiviral (Bystrov, 1975), antidepressant & anxiolytic (Duke, 1992)] support of the common uses in Europe for treating infections and depression. This was determined in the following by different groups either by biotesting individual components or the compound class enriched fractions, which contains the bulk of the hypericins and flavonoids.

Herb Materials: an alcoholic tincture of St. John's Wort (*Hypericum perforatum*) raw material was purchased from a commercial source.

Prep-CC Method: Approximately 14 ml of the commercial alcoholic tincture was evaporated to dryness overnight to yield 640 mg of residue. The resultant extract was triturated with four parts of SiO_2 (silica gel) and was

layered onto a preparative open glass column prepacked with 30 g of SiO_2. The CC (column chromatography) conditions were as follows: column-SiO_2; flow rate 133 ml/hr; five fractions (200 ml) were collected with the following elution profile using: $CHCl_3$/MeOH 8:2 (two fractions made up of distinctly colored bands); $CHCl_3$/MeOH 7:3, $CHCl_3$/MeOH 1:1 and 100% MeOH.

The liquid extract (SJ041, 14 ml) was evaporated to dryness under vacuum to yield 0.64 g of residue. The dried residue was triturated (intimately mixed) with four parts of Silica gel and sifted on top of a glass column pre-packed with 30 g of Silica gel in chloroform. Development of the column over a six-hour period was accomplished by step gradient using chloroform: methanol. In the elution process, 200 ml of solvent volume was employed for each step, with the ratio being changed from 8:2, 7:3, 1:1, and finally 100% methanol. Fractions 1 and 2, consisting of two distinctive colored bands, were obtained from the ratio 8:2 $CHCl_3$:MeOH eluent. The other fractions represent the eluates from each subsequent gradient elution. The collected fractions were evaporated to dryness under vacuum and their yield given below:

eluent solvent (chloroform:methanol)		fraction No.	weight (mg)
ratio	volume (ml)		
8:2	200	SJ041-1	56.9
		SJ041-2	60
7:3	200	SJ041-3	150.3
1:1	200	SJ041-4	49.8
methanol	200	SJ041-5	115.1

TLC Analysis:

The TLC chromatogram showed a text-book separation of the five fractions listed above. Reference standards of contained phytochemicals were not co-chromatographed. The chemical content of the fractional analysis is shown in FIG. 10.

9.5. BIOLOGICAL ACTIVITY ANALYSIS MONOAMINE OXIDASE, SEROTONIN TRANSPORTER ASSAY, OTHER ASSAYS

9.5.1. MONOAMINE OXIDASE-A (MAO-A)

Monoamine oxidase (MAO, E.C.1.4.3.4) is a much less discriminating enzyme, in that it will catalyze the removal of an amine group from a variety of substrates, including endogenous substances (norepinephrine, epinephrine, dopamine, tyramine, 5-hydroxytryptamine) and many drugs that are amines. MAO functions as an important protective mechanism against exogenous, biologically active amines. There are at least two types of MAO, which display dissimilar preferences for substrates and differential sensitivities to selective inhibitors; these were originally defined by sensitivity to clorgyline and preference for 5-HT (MAO-A), and sensitivity to selegiline (deprenyl) and preference for phenylethylamine (MAO-B). The two types of MAO appear to be distinct molecular entities that exist in various proportions in different tissues. The MAO inhibitors currently in therapeutic use are relatively nonselective, but selective inhibitors may offer advantages in certain clinical settings. Only selective inhibitors of MAO-A (e.g. clorgyline) appear to have efficacy in the treatment of major depression, and a selective MAO-B inhibitor may have a beneficial effect on Parkinson's disease and dyskinesia.

MAO_A enzyme activity is assayed in a mitochondrial fraction isolated from rat brain by differential centrifugation. 40 μg membrane protein in 20 mM phosphate buffer (pH 7.4) is mixed with test compound, and the reaction is started by the addition of 95 μM [^{3}H]serotonin (2-10 mCi/mmol). Following 20 minutes incubation at 37°C, the reaction is terminated by the addition of 5% HCl. The radioactivity of [^{3}H]serotonin in an extraction is determined. Compounds are screened at 10 μM. (Medvedev et al., 1994, *Biochem. Pharmacol.* 47: 303-308).

Inhibition of Monoamine Oxidase A Activity

Compound	IC_{50} (μM)
Tetrindole Mesylate	0.046
Pirlindole Mesylate	0.016
*Clorgyline	0.00089

* Standard reference compound

The results of the monoaminooxidase A assay are described in the summary tables below.

9.5.2. MONOAMINE OXIDASE B

Monoamine oxidase (MAO, E.C.1.4.3.4) is not a very discriminating enzyme and will catalyze the removal of an amine group from a variety of substrates, including endogenous substances (norepinephrine, epinephrine, dopamine, tyramine, 5-hydroxytryptamine) and many drugs that are amines. MAO functions as an important protective mechanism against exogenous, biologically active amines. There are at least two types of MAO, which display dissimilar preferences for substrates and differential sensitivities to selective inhibitors; there were originally defined by sensitivity to clorgyline and preference for 5-HT (MAO-A), and sensitivity to selegiline (deprenyl) and preference for phenylethylamine (MAO-B). The two types of MAO appear to be distinct molecular entities that exist in various proportions in different tissues. The MAO inhibitors currently in therapeutic use are relatively nonselective, but selective inhibitors may offer advantages in certain clinical settings. Only selective inhibitors of MAO-A (*e.g.*, clorgyline) appear to have efficacy in the treatment of major depression, and a selective MAO-B inhibitor may have beneficial effect on Parkinson's disease and dyskinesia.

MAO-B enzyme activity was assayed in a mitochondrial fraction isolated from rat liver by differential

centrifugation. 40 μg membrane protein in 20 mM phosphate buffer (pH 7.4) was mixed with test compound, and the reaction was started by the addition of 140 μM [^{3}H]dopamine (2-10 mCi/mmol). Following 20 minutes incubation at 37°C, the reaction was terminated by the addition of 5% HCl. The radioactivity of [^{3}H]dopamine in an extraction was determined. Compounds were screened at 10 μM (Egashira et al. 1976, *Biochem. Pharmacol.* 25: 2583-2586).

The following reference compounds were used for the inhibition of monoamine oxidase B: reference compounds, (IC_{50} (μM)); N-(2-aminoethyl)-4-chlorobenzamide hydrochloride, (23); N-(2-aminoethyl)-3-iodobenzamide hydrochloride, (1.7); and clorgyline, (0.0027).

9.5.3. SEROTONIN TRANSPORTER ASSAY

This assay measured binding of [^{125}I]RTI-121 to presynaptic sites associated with the uptake of serotonin. Cerebral cortical membranes of male Wistar derived rats weighing 175 ± 25 g were prepared in modified Tris-HCl pH 7.4 buffer using standard techniques. A 5 mg aliquot of membrane was incubated with 10 pM [^{125}I]RTI-121 for 90 minutes at 25°C. Non-specific binding was estimated in the presence of 100 μM clomipramine. Membranes were filtered and washed 3 times and the filters were counted to determine [^{125}I]RTI-121 specifically bound. Compounds are screened at 10 μM (Boja et al. 1992, *Synapse* 12:27-36).

Assay Reference Data: Kd 1.1 nM

Bmax: 322 fmol/mg protein

Specific Binding: 85%

Reference Data:

Compound	IC_{50} (nM)	Ki (nM)	nH
Clomipramine	1,100	1,100	0.5
Quipazine maleate	560	550	0.8

6-NO-Quipazine	3,400	3,400	0.7
Trazodone	5,500	5,400	0.8
Mianserin	6.4	2.1	1.0

The results for the MAO-B and the serotonin transporter assay are described in the summary table below.

St. John's Wort Extract-Biological Assay Results

Standard/Extract/Fraction	MAO	5-HT Reuptake
Hypericin	Negative	4 μM
Hyperoside	Negative	Negative
Rutin	Negative	Negative
Quercetin	1.9 μM	Negative
Quecetrin	Negative	Negative
Magniferin	Negative	Negative
Extract #1	Negative	Negative
Extract #2	Negative	Negative
Fraction 1	Negative	Negative
Fraction 2	Negative	Negative
Fraction 3	Negative	Negative
Fraction 4	Negative	Negative
Fraction 5	Negative	Negative

The biological assays verified the MAO-A activity of quercetin and the serotonin uptake activity of hypericin. However, neither the extracts (FIG. 11) nor the fractions (FIG. 10) contained sufficient quantities of either standard (quercetin, hypericin) to detect the two bioactivities. Therefore it was not possible to calculate the percent contribution of each of these components.

9.5.4. OTHER *IN VITRO* STUDIES

While previous studies report that hypericin inhibits MAO at concentrations of 50 g/ml (*e.g.*, Suzuki et al., 1984, *Planta Medica*. 50:272-274), others have failed to confirm

this effect (Bladt and Wagner, 1994, *J. Geriatric Psychiatry Neurology* 7:S57-59; Demisch et al., 1989, *Pharmacopsychiatry* 22:194; Thiede and Walper, 1994, *J. Geriatric Psychiatry Neurology* 7:S54-56). One explanation is that the hypericin used by Suzuki was apparently an extract of only 80% purity. It is possible that one or more constituents of the remaining 20% of this preparation could account for this weak enzyme inhibition. This possibility is supported by Bladt and Wagner (1994) which shows that the St. John's Wort fractions with the greatest MAO inhibition contain the highest concentration of flavonoids. Computer modeling of St. John's Wort constituents also suggests flavonoids to be the most likely MAO inhibitor fraction (Höltje and Walper, 1993, *Nervenheilkunde* 12:339-340); and in a related species (*Hypericum brasiliense*) the xanthone fraction was most active, particularly against MAO-A (Rocha et al., 1994, *Phytochemistry* 36).

However, the MAO inhibition shown for St. John's Wort may not be pharmacologically relevant, since it could not be confirmed *in vivo*. Bladt and Wagner (1994) reported that no MAO inhibition was seen *ex vivo* after administration of 300 mg/kg St. John's Wort extract to rats. Whether this is due to a rapid metabolism of the active constituents or to other reasons cannot be said with certainty at this point. However, pharmacokinetic studies with the St. John's Wort extract, LI 160, showed plasma levels of only 1.5 ng/m of hypericin after a single 300 mg dose and 8.5 ng/ml at steady state in human volunteers (Staffeldt et al., 1994, *J. Geriatric Psychiatry Neurology* 7:S47-53). Unless the active compounds are present in much larger quantities than hypericin, or are concentrated in synaptic terminals, these blood levels are several orders of magnitude below the concentrations needed to inhibit MAO. Inhibition of another degradative catecholamine enzyme, catechol-o-methyltransferase (COMT), can also be seen with various

ethanolic St. John's Wort fractions at super-physiologic concentrations of up to 500 μg/ml (Thiede and Walper, 1994).

Other proposed mechanisms involve effects on serotonin. Müller and Rossel (1994) report that St. John's Wort extract inhibits serotonin receptor expression at 50 μM (~25 μg/ml (according to the way these authors use the term "molarity" when referring to a crude extract) and Perovic and Müller (1995) reported inhibition of serotonin uptake (IC_{50} = 6.2 μg/ml) (Müller and Rossel, 1994, *J. Geriatric Psychiatry Neurology* 7:S63-64; Perovic and Müller, 1995, *Arzneim-Forsch* 45:1145-1148). The concentration required for the former effect could surely never be achieved in the whole animal and even the latter concentration seems unlikely. As a reference comparison, Müller et al. reported an IC_{50} for the synthetic antidepressant, clomipramine, of 0.9 nM (~0.3 ng/ml) for serotonin uptake inhibition (Müller et al., 1996, 2nd International Congress on Phytomedicine, Munich; 1996). In addition, Müller et al. also reported an inhibition of both synaptosomal GABA uptake (IC_{50} = 1 μg/ml LI 160) and $GABA_A$-receptor binding (IC_{50} = 3 μg/ml).

Another novel proposal is that St. John's Wort extract (concentration not provided) reduces cytokine expression [interleukin-6] (Thiele et al., 1994, *J. Geriatric Psychiatry Neurology* 7(suppl. 1):S60-62). The hypothesis is that interleukins can reduce depression in susceptible individuals (Smith, 1991, *Med. Hypotheses* 35:298-306). The field of psychoneuroimmunology is perhaps too new to give a definitive answer regarding this mechanism in the near future, but the link between depression and the immune system is still drawing attention (Kook, et al., 1995, *Biol. Psychiatry* 37:817-819).

Through an NIMH screening contract (NovaScreen™, Baltimore, Maryland) a commercially available crude extract from the fresh flowers and buds of *Hypericum perforatum* [1:1.5; hydro-alcohol (40:60) made from flowering tops; Herb Pharm™] containing ~0.1% hypericin was dried under vacuum,

dissolved in 4% DMSO, and diluted to an initial concentration of 5 g/ml for *in vitro* assay in a battery of 39 receptor types, and two enzyme systems. The receptor assay showing at least 50% displacement of radioligand (or 50% inhibition of MAO) were considered "hits." Concentration-responsive curves (IC_{50}) were then performed for the hits. The results are shown in Table 17.

TABLE 17
NIMH/Nova Screen Receptor Binding/Enzyme Activity Assays Performed with St. John's Wort Extract (Herb Pharm)

Adrenergic ($x_{1,2}$, β) Dopamine ($DA_{1,2}$) Serotonin ($5HT_{1,2}$) Quisqualate Kainate	Glycine (strychnine) $GABA_A$ $GABA_B$ NMDA Adenosine (non-selective)
Angiotensin II Bombesin Substance P Substance K Neurotensin Neuropeptide Y Somatostatin	Arg-Vasopressin 1 $GABA_A$ $GABA_B$ NMDA Adenosine (non-selective) Glycine (strychnine) $GABA_A$
Forksolin	Calcium (N, T, L)
Phorbol Ester Inositol triphosphate (IP_3)	Chloride Potassium
Glycine (non-strychnine) MK-801 PCP Benzodazepine (central BDZ)	 MAO_A MAO_B

The crude extract of St. John's Wort had significant receptor affinity for adenosine, $GABA_A$, $GABA_B$, benzodiazepine, inositol triphosphate (IP_3,) and monoamine oxidase (MAO-A, MAO-B). The inhibition of MAO by crude St. John's Wort extracts is consistent with previous reports (Bladt and Wagner, 1994; Suzuki et al., 1984, *Planta Medica*. 50:272-274; Thiede and Walper, 1994). The results are shown in Table 18.

TABLE 18

Extract Concentration [μg/ml]	% Inhibition in Various Assays							
	Adenosine	$GABA_A$	$GABA_B$	$5HT_1$	BDZ	IP_3	MAO_A	MAO_B
0.005	-2	7	45	12	-4	-3	9	-1
0.05	13	43	85	12	0	16	4	-10
0.5	17	94	105	9	0	13	33	-2
5.0	20	100	109	12	19	40	97	53
50	71	101	114	54	65	107		
$\sim K_i$ (μg/ml)*	1	0.075	0.006	25	24	10	2	3.2

*K_i = IC_{50}/1 + ligand affinity/ligand concentration

Unlike the crude extract, however, synthetic hypericin (95%) lacked significant MAO-A or MAO-B inhibition at concentrations up to 0.1 mM (Table 18). Hypericin had affinity only for NMDA receptors (K_i ~ 1 M) and this may play a role in its reported antiviral activity since NMDA antagonists prevent gp 120-induced neurotoxicity (Diop et al., 1994, *Neuroscience Letters* 165:187-190).

TABLE 19

Receptor Binding/Enzyme Inhibition by Hypericin

Drug conc. (μM)	% Inhibition		
	MAO-A	MAO-B	NMDA
.001	—	—	10.5
.01	—	—	24.5
.1	—	—	-0.3
1	—	—	48.5
10	27.0	-2.0	81.7
100	20.8	-20.7	
$\sim K_i$	—	—	1.1

These data are consistent with recent pharmacologic evidence suggesting that other constituents of this plant may be more important for the reported psychotherapeutic activity. Some of these results have been previously reported (Cott, 1995, *Psychopharm. Bulletin* 31:131-137; Cott, 1996, *Psychopharm. Bulletin* 31:745-751). With the exception of $GABA_A$ and $GABA_H$, the concentrations of St. John's Wort extract required for these *in vitro* activities may not be attained after oral administration in whole animals or humans. It is conceivable that the very high affinity of St. John's Wort extract for GABA receptors presented here may be important. Unless the responsible components are metabolized before they enter the general circulation, plasma levels sufficient to bind GABA receptors would be predicted. It should be noted that Müller, et al., (1996) reported an IC_{50} of 3 μg/ml for $GABA_A$. The reason for this difference with the present finding is unknown.

The significance of this GABA binding is unknown at the present, but there is considerable literature on the role of GABA in affective disorders. $GABA_B$ stimulation has been found to enhance receptor down regulation during imipramine treatment (Enna, et al., 1986, in Bartholini et al. eds. *GABA and Mood Disorders: Experimental and Clinical Research*: Raven Press: New York, NY, 1986:23-49). Nielsen, et al. have reported antidepressant effects with the GABA-ergic agent, fengabine, in depressed outpatients (Nielsen et al. 1990, *Acta Psychiatrica Scandinavica* 82:366-371). Petty et al. have reported that GABA plasma levels are low in both bipolar and unipolar depression, and that benzodiazepines (which enhance $GABA_A$ activity) may be effective antidepressants as well as anxiolytics (Petty et al., 1992, *Biological Psychiatry* 32:354-363; Petty et al., 1993, *Neuropsychopharmacology* 9:125-132; Petty et al., 1995, *Biological Psychiatry* 38:578-591). GABA neuronal systems also modulate dopamine and dopamine-induced behaviors (Cott,

et al., 1976, *Naunyn Schmiedebergs Arch. Pharmacol.* 295:203-209; Cott and Engel, 1977, *J. Neural Transm.* 40:253-268).

9.6. ACTIVE COMPONENTS OF ST. JOHN'S WORT IN THE LITERATURE

St. John's Wort contains numerous compounds with documented biological activity. Most researchers consider its effects to be due to a variety of constituents rather than any single component. Constituents that have stimulated the most interest include the naphthodianthrones, hypericin and pseudohypericin and a broad range of flavonoids including quercetin, quercitrin, amentoflavone and hyperin. Both classes of compounds are reported to contribute to its antidepressant and antiviral activity. The phloroglucinols, hyperforin and adhyperforin, the essential oil, flavonoids and xanthones all contribute to St. John's Wort's wound-healing properties.

9.6.1. ST. JOHN'S WORT COMPONENTS

The major active components of St. John's Wort are naphthodianthrone derivatives (<0.1-0.15%) (Deutscher Arzneimittel-Codex. 3rd Supplement 91 ed. 1986); hypericin [0.02-1.8%] (Benigni et al., Hypericum, Piante Medicinali: Chimica, Farmacologia e Terapia. Inverni & Della Beffa, Milano, 1971.), pseudohypericin, isohypericin, emodin-anthrone. In fresh material protohypericin and protopseudohypericin are also present. These biosynthetic precursors are transformed into hypericin and pseudohypericin by exposure to light. Cyclopseudohypericin is also cited and is an oxidation product of pseudohypericin (ESCOP, 1996, Monograph St. John's wort. European Scientific Cooperative for Phytomedicines).

9.6.2. HYPERICIN CONTENT OF ST. JOHN'S WORT

The highest concentrations of hypericin have been observed in the dried flowers (Benigni, 1971) followed by the capsules and uppermost leaves (Benigni, 1971; Southwell and Campbell, 1991, *Phytochemistry* 30:475-478). Narrow-leafed varieties which have a relatively high number of oil glands [6.2 per mm] are reported to yield significantly higher concentrations of hypericin than broad-leafed varieties [2.2 oil glands per mm] (Southwell and Campbell, 1991).

Hypericin content in various parts of *H. perforatum* in g%

Young plant (12 cm tall)	0.027
Whole plant w/flowers	0.036-0.2
Whole plant w/buds	0.042
Dried flowers	0.196-1.8
Fresh flowers	0.09-0.12
Petals	0.245
Leaves (average)	0.019
Stem	0.021

(Benigni et al., 1971; List and Hörhammer, 1993)

Hypericin content in parts per million (Benigni et al., 1971)

Flower & buds	2150 ppm
Capsules	730 ppm
Top leaves	380 ppm
Bottom leaves	290 ppm
Side stem	120 ppm
Main stem	40 ppm
Narrow-leaf varieties	1040-1630 ppm (6.1 oil glands per mm)
Broad-leaf varieties	370-580 ppm (2.2 oil glands per mm)

(Benigni et al., 1971; Southwell and Campbell, 1991)

9.6.3. FLAVONOID CONTENT OF ST. JOHN'S WORT

Of 223 species of plants tested for flavonoid content, the flowers of *Hypericum perforatum* were the highest at 11.7% (Tsitsina, 1969, *Tr. Bot. Sadov. Akad. Nauk. Kaz.*, 111-114). Among the biflavonoids, the proanthocyanidins consisting of dimers, trimers, tetramers and high polymers represent 12% of the dried weight of the aerial portion of the plant. These include the following flavonols; kaempferol, luteolin, myricetin, quercetin (2%); flavone glycosides; quercitrin (0.524-0.3%), isoquercitrin [0.3%] (Dorossiev, 1985, *Pharmazie* 585-586; Koget, 1972, *Khimiya Prirodnykh Soedinea* 242-243), hyperin [0.7-1.1% hyperoside] (List and Hörhammer, 1993), I3', II8-biapigenin [0.1-0.5%] (Berghöfer and Hölzl, 1987, *Planta Medica* 216-219; List and Hörhammer, 1993) amentoflavone, I3', II8-biapigenin (0.01-0.05% in flowers), rutin [0.3%] (Akhtardzhiev et al., 1984, *Farmatsiya* (Sophia) 34:1-6; Berghöfer and Hölzl, 1989, *Planta Medica*. 91; Kitanov, 1987, *Khimiya Prirodnykh Soedinenii* 2:185-203), gentistic acid, leucocyanidin (Benigni et al., 1971).

Contents of tannins in *Hypericum perforatum* in g%	
Commercial Supplies (Whole plant)	3-12.1
Inflorescence	12.4-16.2
Leaves	12.4
Stems	3.8
Flowers *H. Perforatum* var. *Vulgare*	16.2
Flowers *H. perforatum* v. *angustifolium*	11.1

(Benigni et al., 1971)

Flavonoid and procyanidin concentrations have been reported to be highest in the flowers during budding stage immediately before flowering (11.71%), followed by the leaves

and stems (7.4%). Flavonoid concentrations are also reported to be highest in plants growing in higher altitudes and those growing on Northern slopes where the weight of the plant is lower than plants growing in Southern exposure (Brantner et al., 1994, *Scientia Pharmaceutica* 62:261-276; Tsitsina, 1969; Zhebeleva, 1973, *Rast. Resur.* 9:402-404). The highest concentration of I3', II8-biapigenin occurs in buds and flowers, the fruit has very low concentrations and the stems and leaves no trace (Berghöfer and Hölzl, 1987). Hyperin is highest in the flowers (3%) followed by the leaves (1.05-1.80%) and only traces in the stems [0.13%] (Maksyutina and Koget, 1971, *Khimiya Prirodnykh Soedinenii*, 3:363-367). Quercetin is found in the leaves and flowers (0.1-0.582%) with trace amounts in green leaves, higher amounts in red colored leaves, the highest amount in the leaves during flowering, and still higher amounts in the flowering tops. Rutin is found in all parts but is much higher in the leaves (2%) during the budding stage than in the flowers (0.095%), is higher in plants growing in dry vs. those growing in moist conditions, and is reported to be highest when harvested in the evening.

Sun-macerated preparations yield higher amounts of rutin than preparations macerated in the dark (Benigni et al., 1971). In one analysis of fresh material quercetin was found to be higher in the top half of the plant than in the leaves only or flowering tops only (Smith et al., 1996, Quality Validation Laboratory - Herb Pharm™:Williams, OR, 1996).

Flavonoid content in various parts *H. perforatum* in g%		
Constituent	Plant Part	Concentration
Total Flavonoids	flowers	11.7
Total Flavonoids	stems and leaves	7.4
Quercetin	leaves and flowers	0.1-0.582
Quercitrin	whole herb/flowers	0.524-0.3
Isoquercitrin		0.3
Hyperin (hyperoside)		0.7-1.1
I3, II8-biapigenin	fresh flowers	0.1-0.5
I3, II8-biapigenin	stems and leaves	non-detectable
I3', II8-biapigenin (amentoflavone)	flowers	0.01-0.05
Rutin	flowers	0.095
Rutin	leaves	2

(Akhtardzhiev et al., 1984; Benigni et al., 1971; Berghöfer and Hölzl, 1989; Brantner et al., 1994; Dorossiev, 1985; Kitanov, 1987; Koget, 1972; List and Hörhammer, 1993; Smith et al., 1996; Tsitsina, 1969)

9.6.4. ESSENTIAL OIL FROM ST. JOHN'S WORT

The essential oil consists predominantly of monoterpenes (pinenes) and sesquiterpenes and constitutes 0.1-1% (Benigni, 1971; ESCOP, 1996). The primary compounds include the saturated hydrocarbons methyl-2-octane (16.4%) and α-pinene (10.6%); also present are traces of methyl-2-decane, methyl-2-butenol and undecane, α- and β-pinene, α terpineol, geraniol, traces of myrcene, limonene, caryophyllene, humulene, C_{16} and C_{24} n-alkanes, C_{24}, C_{26} and C_{28} n-alkanols (Brondz and Greibrokk, 1983, *Journal of Natural Products* 46:940-941; Brondz et al., 1983, *Phytochemistry* 22:295-296; Mathis and Ourisson, 1964, *Phytochemistry* 3:37-378).

Essential oil content in the stem is very small, and is greater in the mature capsule. It is also richer before flowering (0.26%) than when in flower (0.11%). When the stem is eliminated the plant yields an average of 0.35% essential oil (Benigni et al., 1971).

9.6.5. PHLOROGLUCINOLS IN ST. JOHN'S WORT

Hyperforin (prenylated derivative of phloroglucinol), adhyperforin (similar to the bitter principle of hops, adhumulone). Hyperforin and adhyperforin levels increase considerably during the formation of the fruits with hyperforin increasing from 2.0% in the flowers to 4.5% in the fruits based on dry weight, and polar hyperforin-like compounds increasing from 0.05-0.3%. Adhyperforin increased 10-fold from 0.2% in the flowers to 1.9% in the capsules (Benigni et al., 1971; Brondz et al., 1982, *Tetrahedron Letters* 23:1299-1300; Bystrov, 1975, *Tetrahedron Letters* 32:2791-2794; Maisenbacher and Kovar, 1992, *Planta Medica* 291-293). The hyperforins are lipophilic and unstable when exposed to heat and light.

9.6.6. MISCELLANEOUS COMPOUNDS IN ST. JOHN'S WORT

Choline, carotenoids (lutein, violaxanthin, cis-throllixanthin, throllichromone), beta-sitosterol, pectin, phlobaphene and rhodan; caffeic (0.1%), chlorogenic, isovalerianic, lauric, myristic, nicotinic (0.12% in leaves), palmitic and stearic acids; amino acids including cysteine, GABA (0.7 mg/g), glutamine, leucine, lysine, ornithine, proline, threonine; scopoletin, umbelliferone; vitamin C, xanthonolignoids (1.28 mg/100g, kielcorin) (Bennett and Lee, 1989, *Phytochemistry* 28:967-998; Karryev, 1980, *Izv. Akad. Nauk. Turkm. SSR.* 52-57; List and Hörhammer, 1993).

Activity of Constituents	
Adhyperforin	Antibacterial (Bystrov, 1975); neurotransmitter inhibitor, potential anticarcinogenic (Oittmann et al., 1971, *Arzneim-Forsch.* 21:1999-2000)
Amentoflavone (I3', II8-biapigenin)	Anti-inflammatory, antiulcerogenic (Berghöfer and Hölzl, 1989)
Flavonoids	Analgesic (Vasil'chenko et al., 1986)
Flavonols	Sedative (Berghöfer and Hölzl, 1987)
GABA	Sedative
Hyperforin	Antibacterial against gram positive bacteria; wound-healing (Bystrov, 1975; Maisenbacher and Kovar, 1992)
Hypericin	Antiviral (Lavie et al., 1995), anxiolytic (Holly and Strowski)
Methyl-2-butenol	Sedative
Proanthocyanidins	Antioxidant, antimicrobial, antiviral, vasorelaxant
Xanthones	Antidepressant, antimicrobial, antiviral, diuretic, cardiotonic, MAO_A inhibitor (Kitanov and Blinova, 1987)

9.6.7. ANALYTICAL METHODS OF ANALYSIS

Two compounds of interest as marker constituents include two naturally occurring pigments, hypericin and pseudohypericin (naphthodianthrones). These dyes are characteristic markers for this herb and are easily extracted into methanol. They both absorb visible light with a maximum absorption at 588 nm and are highly fluorescent in methanol. Both pigments are similar in their absorption and emission spectra, including their absorbtivity. Separation of these two pigments is necessary to determine the concentration of each pigment. Flavonoids are also considered to be an important class of constituents in St. John's Wort. Methods are provided for both classes of compounds.

9.6.8. THIN LAYER CHROMATOGRAPHY (TLC) OF HYPERICIN AND PSEUDOHYPERICIN

Sample Preparation

An extract of hypericin is prepared from a representative sample of dried plant material by repetitively extracting 1.0 g of the sample with four successive 10 ml portions of methanol. The entire contents of all four extractions are diluted to 50 ml in a volumetric flask. This solution is filtered through a 0.45 μm filter prior to analysis by TLC or HPLC.

Standard Preparation

Synthetic hypericin standard (ICN Biochemical™, Cleveland, OH, Cat # 193423) is dissolved in pure methanol at a concentration of 0.10 mg/ml and filtered through a 0.45 μm filter. This standard stock solution is used for both TLC and HPLC calibration.

Stability of Standards/Sample Solutions

Both hypericin and pseudohypericin pigments, as well as solutions prepared from them are stable for months if kept in the dark.

Stop Test:

A small spot of the above extracts on filter paper will exhibit bright red fluorescence under UV-365 nm light. The liquid extract solutions is also fluoresce bright red under UV-365 nm light.

Chromatographic Conditions

The chromatographic conditions for TLC are typically as follows. Silica Gel (*e.g.*, Eastman No. #6060-13181 with or

without fluorescent indicator added to the gel) is developed with toluene:ethyl acetate:glacial acetic acid in the proportions of 3:6:1. Typically, 1-5 μl of sample is added via capillary. The detection is performed with UV light at 365 nm. Hypericin pigments also fluoresce bright red so visible light detection may be used. The Rf values for hypericin are 0.71 and for pseudohypericin 0.50. The detection limits for this assay are 0.2 μg.

9.6.9. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF HYPERICIN AND PSEUDOHYPERICIN

The sample presentation and standards were prepared as described in the TLC experiment above.

The HPLC parameters are as follows. A Waters Nova-pak™ C-18 column, 3.9 X 150 mm is developed with a mobile phase under isocratic conditions where the mobile phase is methanol:0.4% phosphoric acid:triethylamine (82:17:1). The flow rate is 1.0 ml per minute and detection is performed using a visible detector at 588 nm. The column is run at ambient temperature. Run time is typically 12 minutes for injection of 10-50 μl of sample. The elution rates of the active components pseudohypericin and hypericin are 2.8 and 9.6 minutes, respectively.

Limits of Quantitation and Detection

Limits of detection depend upon instrumentation. For our use a Waters Mod I™ HPLC system with a Waters 991M Photodiode Array (PDA) detector and monitoring at 588 nm was used. The detection limits are approximately 2.5 μg for both pseudohypericin and hypericin.

Spike Recovery Validation

Spike recovery is typically 100% (+/-5%) when total absorbency of spiked sample is less than 0.8 AUFS.

HPLC (Flavonoids, Hypericin and Pseudohypericin) Extraction

After filtration, the residue is extracted with 40 ml acetone at room temperature for 10 minutes using an ultrasonic bath and filter. The methanol and acetone filtrates are combined and reduced to dryness under vacuum. The dry material is dissolved in 4.0 ml methanol and filtered.

The HPLC for flavonoids, hypericin and pseudohypericin is run under the following conditions.

A LichroCart™ reverse phase (RP) 18 supersphere column, 4 x 250 mm with a reverse phase (RP) 8 pre-column is developed at ambient temperature with a flow rate of 1 ml/minute for minutes 0-39 and after 40 minutes 0.6 ml per minute. The injection volume is 20 µl and detection is at UV 254 nm.

Three different mobile phases are used. To detect rutin, hyperoside and isoquercitrin, acetonitrile:water:phosphoric acid (16:83:1) is used with a run time of 30 minutes.

For detecting quercitrin and isoquercetin, the following system is used, acetonitrile:water:phosphoric acid (32:67:1) with a run time of 45 minutes.

For I3, II8-biapigenin, amentoflavone, pseudohypericin and hypericin the conditions are as follows. The solvent system is acetonitrile:methanol:water:phosphoric acid (55:20:24:1). The run time is 75 minutes.

The retention times (in minutes) are as follows: rutin (16.7); hyperoside (18.5); isoquercetrin (19.2); quercetin (23.8); luercetin (36.4); I3, II-biapigenin (42.8); amentoflavone (55.9); pseudohypericin (59.7); and hypericin (68.4).

Standards for rutin, hyperoside, isoquercitrin, quercitrin and hypericin is available from Sigman, St. Louis, MO, USA, with the rest available from Roth. The samples are

quantified for the procedure of Kartnig et al., 1996, *Planta Medica* 62:51-53 against an external standard in methanol at 25 μg/ml.

9.6.10. UV/VIS SPECTROSCOPIC METHOD (HYPERICIN/PSEUDOHYPERICIN) SAMPLE PREPARATION

A quantitative extract is prepared by extracting 1.0 g of powdered herb sample with three 25 ml portions of dichloromethane (CH_2Cl_2) or until filtrate is clear. The dichloromethane extract is discarded. The dried residue is extracted exhaustively with acetone. The acetone extract is evaporated to dryness under vacuum. The residue is dissolved in three 8 ml portions of methanol and transferred to a 25 ml volumetric flask. Enough additional methanol is added to make the total volume 25.00 ml. 10 ml of this solution are filtered; the first 2 ml are discarded. 5.0 ml of the filtrate is diluted to 25.00 ml with methanol in a separate 25 ml volumetric flask.

9.7. HPLC ANALYSIS OF ST. JOHN'S WORT COMPONENTS HYPEROSIDE, RUTIN, QUERCETIN, QUERCITRIN, HYPERICIN, MANGIFERIN

The analytical HPLC method was as follows. The HPLC system was a Waters™ HPLC system consisting of two model 510 EF pumps, model 717 autosampler, a model 486 UV-Vis detector set at 254nm, and a Millenium™ Version 2.15 system controller and data processing software. Separations were made using 10 μl injection volumes loaded onto a reversed-phase C-18 column (Beckman Ultrasphere™, ODS column, 5μum, 250 x 4.6 mm) and a gradient elution system, using eluents A and B (A = 0.1 M NaH_2PO_4 in 0.05% TFA H_2O; B = ACN) according to the following profile: 0-10 minutes 100-60% A, 0-40% B; 10-20 minutes 60-0% A; 40-100% B; 20-30 minutes 0-100% A, 100-0%B. The flow rate was kept at 1.0 ml/min., with peak monitoring at 254 nm.

Magniferin, rutin, hyperoside, quercetrin, quercetin and hypericin components were determined. The results for five commercially available samples were as follows:

Component Name	SJ049	SJ042	SJ045	SJ048	SJ039
Hyperoside	5.750	2.660	4.160	6.310	24.100
Rutin	4.790	2.280	5.500	6.740	12.000
Quercetin	0.830	0.650	0.940	1.360	3.270
Quercitrin	0.910	0.830	1.050	1.040	1.560
Hypericin	0.370	0.140	0.160	0.110	0.490
Mangiferin	0.150	0.260	0.160	0.015	0.020
TOTAL FOR COMPONENTS STUDIED	12.64%	6.22%	11.46%	14.32%	41.54%

The results are also represented in FIG. 11.

10. EXAMPLE: GINGER, *Zingiber officinale*

Ginger may be prepared by a variety of methods. It may be sliced and freeze-dried and pulverized. It may be steamed and distilled to obtain the essential oil. It may be solvent extracted.

10.1. PLANT SOURCE/EXTRACTION METHOD:

Further methods of processing and extracting natural ginger may be found in the methods of processing and extracting section of the detailed description.

10.2. COMMERCIAL SUPPLIERS/PRODUCT NAMES

Ginger is also commercially available from a variety of sources, including Hauser Nutraceuticals (Boulder, CO), Herb Phyters (West Hollywood, CA), Nature's Fingerprint Triarco Industries, Inc., distributed by GNC, Natures Way (Springvale, UT), Solaray (Ogden, UT) and Zintona (Israel). Herbal Choice-Botalia, Herb Pharm, Nature's Resource.

10.3. CLINICAL UTILITY

The clinical use of ginger is for motion sickness and treating nausea and vomiting. Ginger appears to alleviate motion sickness by acting on the gastrointestinal tract, rather than acting through the central nervous system as antihistamines act. Some authors have proposed that it may increase gastric motility, blocking gastrointestinal reactions and subsequent nausea feedback, others have found no evidence (Mowrey and Clayson, 1982, *Lancet* 655).

Ginger also ameliorates menstrual disorders and other disorders associated with nausea or gastrointestinal discomfort. Another use of ginger is preventing post-operative nausea and vomiting (Arfeen et al., 1995, *Anaesth. Intens. Care* 23:449-454). Others have reported ginger showing antiplatelet activity, preventing mucosal damage, a hypocholesterolemic activity, cardiovascular activity, cardiotonic activity, anti-inflammatory activity and antipyretic activity (Mustafa et al., 1993, *J. Drug Dev.* 6(1):25-39). Mustafa also report that ginger acts through the thromboxane B_2 levels in humans. Other workers have investigated that ginger's activities through prostacyclin (PGE2) and thromboxane (TXA_2) moieties (Bakon, 1986, *Med. Hypothesis* 20:271-278).

10.4. FRACTIONAL ANALYSIS PREPARATIVE HPLC

The fractionation of alcoholic raw ginger extract was performed using reversed-phase preparative HPLC. This method was selected as a preferable prep-chromatographic technique on the basis of observed excellent mass recovery (>90%), the separation of the selected standards (shogaols and gingerols), as well as separation of the other co-ocurring components. A detailed description of the materials and methods utilized is described below.

The selection of chemical markers for ginger was based on the following protocol. A comprehensive search of the

literature on ginger (*Zingiber officinale*) indicated the 6-, 8- and 10-gingerols, as well as the 6-, 8- and 10-shogaols as the components with the most consistent bioactivity in a large number of diverse assays [analgesic, anti-emetic, anti-5-HT, cardiotonic, prostaglandin and thromboxan suppression (Duke, 1992)]. The non-CNS anti-emetic clinical indication is supported by the prostaglandin/thromboxan bioactivities, as this pathway plays a key role in the gut (regulation of acid production/absorportion/membrance regeneration). These biodata were determined by different groups either by biotesting individual components or the essential oil extract of ginger, which contains the bulk of the gingerols and shogaols.

Herb Materials: one bag of powdered dry ginger (*Zingiber officinale*) raw material was obtained from Hauser Nutraceuticals, and labeled as LIMS 157381.

Prep-HPLC Method: Approximately 150g of dry ginger powder was suspended in 500 ml of 95% MeOH/H_2O and mixed overnight. The resultant extract was filtered and rotary evaporated to an oily residue, and 10 ml of residue was injected onto a preparative HPLC(SepTech NavaPrep 5000™). The HPLC conditions were as follows: column-Vydac C18, 5.0 x 25 cm ID; UV-detector @ 282 nm; flow rate 100 ml/min.; gradient from 10% ACN to 100% ACN over 30 minutes, followed by a 10 minute hold at 100% ACN. Fractions (300 ml) were collected every three minutes for the first thirty minutes, followed by a 1 liter fraction collected from 30-40 minutes for a total of 11 fractions.

Analytical HPLC Method: The HPLC system was an Hitachi HPLC system consisting of an L-4500A Diode Array Detector, and D-6000 Interface, L-6200A Intelligent Pump and AS4000 Intelligent Autosampler™. Separations were made using 10ul injection volumes loaded onto a reversed-phase C-18 column (Alltech Hypersil™ ODS, 5μm, 4.6-260mm) and a gradient elution system, using eluents A and B (A = ACN; B = 99/1 H_2O/MeOH) according to the following profile: 0-5 minutes 55 %

ACN, 45 % MeOH/H_2O; 14 minutes 80 % ACN, 20 % MeOH/H_2O; 20-25 minutes 55 % ACN, 45 % MeOH/H_2O; 0-5 minutes 55 % ACN, 45 % MeOH/H_2O; 0-5 minutes 55 % ACN, 45 % MeOH/H_2O. The flow rate was kept at 1.0 ml/min., with peak monitoring at 282 nm. The reproducibility of this analytical method should not exceed 2.5% relative standard deviation.

The fractionation of alcoholic raw ginger extract was performed using reverse-phase preparative HPLC. This method was selected as a preferable prep-chromatographic technique on the basis of observed excellent mass recovery (>90%), the separation of the selected standards (shogaols and gingerols), as well as separation of the other co-occurring components. A detailed description of the materials and methods utilized is described below.

Approximately 150 g of dry ginger powder was suspended in 500 ml of 95% methanol in water and mixed overnight. The sample extract was then rotary evaporated to an oily residue, and 10 ml of this residue was injected onto a preparative HPLC. The HPLC conditions were as follows: column-Vydac C18, 5.0 x 25 cm ID, cat number 218TP101550; detector-282 nm; flow rate 100 ml/minutes; gradient from 10% acetonitrile to 100% acetonitrile over 30 minutes followed by a 10 minute hold at 100% acetonitrile. Fractions were collected every 3 minutes for the first 30 minutes followed by a 1 l fraction collected from 30 to 40 minutes for a total of 11 fractions.

A comprehensive search of the literature on ginger (*Zingiber officinale*) indicated the 6-, 8- and 10-gingerols, as well as the 6-, 8- and 10-shogaols as the components with the most consistent bioactivity in a number of assays (analgesic, anti-emetic, anti 5-HT, cardiotonic, prostaglandin suppressor; see Duke, 1992). The choice amongst the compounds in the literature was determined in the following by different groups either by biotesting individual components or the essential oil extract of ginger, which contains the bulk of the gingerols and shogaols.

The fractions were then analyzed by HPLC as above for the determination of 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, 8-shogaol, and 10-shogaol. Quantification for all components was performed based upon the response of a capsaicin standard purchased from Sigma Chemical Co.™ The standards for the ginger chemical analysis are available through literature methods. For example, several reviews exist and sources may be found in the following: (Gvindaragan, 1982, *CRC: Critical Reviews in Science and Nutrition* 17:191-96, 189-256; Van Beek et al., 1987, *Phytochemistry* 26:3005-3010).

Results for each fraction are listed in Table 20. The results are represented in FIG. 12.

TABLE 20

Results of Ginger Fractionation

Ginger:	% w/w						
	6-gingerol	8-gingerol	10-gingerol	6-shogaol	8-shogaol	10-shogaol	Totals:
Fraction 1	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fraction 2	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fraction 3	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fraction 4	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fraction 5	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Fraction 6	1.060	0.000	0.000	0.000	0.000	0.000	1.060
Fraction 7	0.170	0.160	0.000	0.000	0.000	0.000	0.330
Fraction 8	0.020	0.060	0.120	0.410	0.000	0.000	0.610
Fraction 9	0.010	0.000	0.200	0.000	0.070	0.000	0.280
Fraction 10	0.000	0.000	0.000	0.000	0.000	0.000	0.100
Fraction 11	0.000	0.000	0.000	0.000	0.000	0.000	0.000

After HPLC analysis, each fraction was evaporated to an oily residue and the weight of the residue was determined.

10.5. BIOLOGICAL ACTIVITY ANALYSIS INHIBITS THROMBOXANE SYNTHESIS, PROSTACYCLIN AGONIST; PG SYNTHETASE INHIBITOR; SPECIFIC BIOASSAY THROMBOXANE SYNTHETASE INHIBITION

10.5.1 THROMBOXANE A_2 BIOASSAY

Thromboxane A_2 (TxA_2) is a potent platelet activator which serves to amplify the response to weak platelet agonists, such as ADP, epinephrine, and low concentrations of collagen and thrombin. TxA_2 is generated by cyclooxygenase from arachidonic acid, itself released by phospholipases upon cell activation. The resulting unstable prostaglandin endoperoxides (PGG_2 or PGH_2) are converted to TxA_2 by the enzyme TxA_2 synthase. The endoperoxides and TxA_2 generated by aggregating platelets could produce a profound vasoconstriction largely restricted to the area of the vessel immediately adjacent to the platelet plug. Vasospasm associated with coronary or cerebral infarction could be produced by potent vasoconstrictors generated during platelet clumping. An enhanced production of TxA_2 is associated with the pathogenesis of various thrombotic and ischemic disorders. The inhibitors of TxA_2 biosynthesis should have beneficial advantages to the ischemic heart diseases, cerebral infarction and arteriosclerosis and diabetic disorders.

Thromboxane A_2 synthase in microsomal fractions used in the assay is isolated from rabbit platelets by conventional centrifugation methods. The reaction is initiated by the addition of substrate, prostaglandin G_2 to the tube which contains test compound (or vehicle), assay buffer and enzyme, and continuously incubating for 30 minutes at 30°C, and then determined the production of thromboxane A_2, which is rapidly converted to thromboxane B_2. The amount of thromboxane B_2 formation is quantitated by radioimmunoassay method. Compounds are screened at 100 μM (Gresele et al., 1991, *Trends Pharmacol. Sci.* 12:158-163; Brownlie et al., 1993, *Brit. J. Pharmacol.* 110:1600-1606).

The results of the thromboxane assay were as follows:

Ginger Extract-Biological Assay Results

Standard/Extract/Fraction	Thromboxane
6-Gingerol	Negative
8-Gingerol	N.T.
10-Gingerol	N.T.
6-Shogaol	N.T.
8-Shogaol	N.T.
10-Shogaol	N.T.
Extract	Negative
Fraction #1	Negative
Fraction #2	Negative
Fraction #3	Negative
Fraction #4	Negative
Fraction #5	Negative
Fraction #6	Negative
Fraction #7	Negative
Fraction #8	Negative
Fraction #9	Negative
Fraction #10	Negative
Fraction #11	Negative

N.T. = not tested

The negative results of the extract, the standard and the fractions, in the thromboxane assay indicate that this assay may be too selective (the mechanism is too narrow) and may not be an appropriate assay for pharmaprinting ginger. A more general assay for probing prostaglandin-related bioactivities of ginger extracts, fractions and standards is the prostanoid FP assay. The details of this tissue assay are described below.

10.5.2. PROSTANOID FP ASSAY

A strip of the uterus obtained from Long Evans derived female rats weighing 275 ± 25 g and sacrificed by cervical dislocation is used. The tissue is placed under 1 g tension in a 10 ml bath containing phosphate buffered saline (pH 7.4) at 32°C. Test substance (30 μM)-induced isometrically recorded contraction by more than 50% within 5 min, relative to control 0.1 μM PGF2α-induced response, indicates possible prostanoid FP receptor agonist activity. At a test substance concentration where no significant agonist activity is seen, ability to reduce the PGF2α-induced contractile response by more than 50% indicates prostanoid FP receptor antagonist activity. (Pettibone et al., 1991, *J. Pharmacol. Exp. Ther.* 256: 304-308; Vane et al., 1973, *Br. J. Pharmacol.* 48: 629-639).

Agonist	EC_{50} (μM)
*Prostaglandin F2α (PGF2α)	0.0012

10.6. CHEMICAL ANALYSIS HPLC FOR 6-GINGEROL, 8-GINGEROL, 10-GINGEROL, 6-SHOGAOL, 8-SHOGAOL, 10-SHOGAOL

The results of the chemical analysis from several commercial suppliers of ginger are below. The results are represented in FIG. 13.

Ginger	mg/capsule				
	GG631	GG635	GG641	GG639	GG634
6-gingerol	1.8	3.3	1.4	2.7	1
8-gingerol	3.6	0.6	0.19	0.48	0.
10-gingerol	0.48	0.9	0.44	0.73	0.
6-shogaol	0.85	0.94	0.59	1.4	0.
8-shogaol	0.44	0.16	0.15	0.33	0.
10-shogaol	0.22	0.08	0.16	0.33	0.
Totals Pungents:	4.15	5.98	2.93	5.97	3.

11. EXAMPLE: CORIOLUS VERSICOLOR

11.1. ISOLATION OF COMPONENTS OF CORIOLUS VERSICOLOR

Coriolus versicolor is a mushroom. The extract of thin mushroom has been used for the treatment of post-operative malignancies. It is believed that the activity of *Coriolus versicolor* is due to immunomodulatory effects.

650 mg of crude Coriolus versicolor powder polysaccharide peptide (PSP) (Landford, 18 capsules lot #941 23 1) were added to 45 ml of a buffer C (see Example 11) and stirred at 4°C overnight. The next day, the solution was centrifuged (10,000 r.p.m., 1 hour) and the supernatant was removed and passed through a series of sugar specific columns (flow rate 6 ml per hour). The columns were washed with buffer C to remove all unbound proteins. Then each column was washed with a buffer D (see mistletoe section) containing EDTA (10 column volumes). These eluates were separately collected and each contained carbohydrate binding proteins which require Ca^{++} for their binding specificities.

Then each column was washed with buffer C and then separately eluted with buffer C containing 0.5 M sugars (lactose, galactose and melibiose). Each eluate was collected and each contained carbohydrate binding proteins which do not require Ca^{++} for their binding specificities.

All six eluates were concentrated using 5K cutoff ultramembrane filters to remove salts and low molecular weight components.

The biological activities of each protein and the materials which did not bind to the columns were assayed in the same leukemia L1210 system in culture as was used in the preceding examples. The protein contents of each sample were assayed by Bio Rad and molecular weights of samples were assayed by 6.5% SDS gels.

The proteins isolated 13.87mg yield = (13.87x100)/605 = 2.29%

The quantitative and bioactivity protein fingerprints for the sample of Coriolus versicolor are set forth in Table 21. The standard fingerprints which were established for pharmaceutical grade are set forth in Table 22. A comparison of the fingerprints for the sample and standard shows that they match, so the sample qualifies as pharmaceutical grade in accordance with the present invention.

TABLE 21

QUANTITATIVE AND BIOACTIVITY FINGERPRINT OF CORIOLUS VERSICOLOR SAMPLE

Fraction identity	Protein Content (mg/ml)	Total Volume (ml)	Total Protein (mg)	IC_{50}[a] (μg/ml)	Total Activity Units[b]
PSP	13.4	45	603	-	-
EDTA Eluted Proteins					
Lactose	0.430	5.0	2.15	0.0600	3.5×10^4
Galactose	0.240	6.0	1.44	0.0210	6.8×10^4
Melibiose	0.480	6.5	3.12	0.0033	91.0×10^4
0.5 M Sugar Eluted Proteins					
Lactose	0.370	8.5	3.14	0.028	11.0×10^4
Galactose	0.459	7.5	3.44	0.110	3.1×10^4
Melibiose	0.074	6.0	0.44	0.150	0.2×10^4
Unbound sugars	3.950	136.0	537	-	-

[a] Inhibitory concentrations (μg/ml) which caused 50% inhibition of the growth of L1210 leukemia cells in culture.

[b] Activity unit is defined as dilution factor needed for a specific fraction which when added to L1210 cells causes a 50% growth inhibition.

TABLE 22

STANDARD QUANTITATIVE AND BIOACTIVITY FINGERPRINT OF CORIOLUS VERSICOLOR

Fraction Identity	Protein Content (mg/ml)	IC_{50}[a]	Total[b] Activity Units
PSP	-	-	-
EDTA Eluted Proteins			
Lactose	0.2 - 0.6	0.01 - 0.10	1.0 - 10 x 10^4
Galactose	0.1 - 0.4	0.01 - 0.10	5.0 - 20 x 10^4
Melibiose	0.3 - 0.6	0.001 - 0.01	50 - 150 x 10^4
0.5 M Sugar Eluted Proteins			
Lactose	0.1 - 0.5	0.01 - 0.10	5.0 - 20 x 10^4
Galactose	0.3 - 0.6	0.1 - 0.5	1.0 - 10 x 10^4
Melibiose	0.01 - 0.10	0.1 - 0.5	0.1 - 5.0 x 10^4
		-	-

[a] Inhibitory concentrations (μg/ml) which caused 50% inhibition of the growth of L1210 leukemia cells in culture.

[b] Activity unit is defined as the dilution factor needed for a specific fraction which added to L1210 cells in culture causes 50% growth inhibition.

12. EXAMPLE: ALOE, *Aloe Vera*

12.1. PLANT SOURCE/BACKGROUND

Aloe has two primary uses. One is use for wound healing and as an anti-inflammatory. The second major use is as a stimulant laxative. It is known as *aloe vera* or a *barbadnsis*. The aloe for wound healing is a mucilaginous gel obtained from the center of the leaf. The aloe form for use as a laxative is obtained from specialized cells that occur on the border of the outer and inner layers of the mesophyll located beneath the epidermis of the leaves of the plant. The laxative form is made from a bitter yellow latex obtained from the plant that is dried to a reddish-black mass. The two aloe forms are very different.

Additional uses for *aloe vera* gel or *aloe vera* extract are for liver protection, arthritis, use as an anti-inflammatory, use as an anti-pruritic, use as anti-bacterial agent, use as an anti-ulcer agent. Amongst the reference books describing aloe are Leung and Foster, 1996, and Bisset, 1994. Aloe has been approved as the subject of the Germany E Monograph in the dried laxative form (Germany Commission E Monograph B Anz. No. 154, dated August 21, 1985.

12.2. COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are many sources for aloe. It is sold commercially in a laxative form by Natures Way Products, Inc. (Springville, Utah). Commercially available gel formulations include *aloe vera* (Aloecorp), Aristo™ (Steiner, Germany), Krauterlax™ (Dolorgiet, Germany) and Dermaide Aloe™ (Dermaide Research Corp.). Stabilized aloe gel compositions have been reported (U.S. Patent No. 3,878,197, Maret).

12.3. CLINICAL INDICATIONS

The major clinical indications for aloe were discussed above, namely, wound healing, anti-inflammatory, liver protection, anti-pruritic, laxative, anti-bacterial and anti-ulcer activity. An additional indication for which aloe may be effective is as an anti-leukemic agent which has been demonstrated *in vitro* (Kupchan, 1976, *J. Nat. Prod.* 39:223). Others have reported activity against peptic ulcers and also improved circulation in minimal studies. The FDA studied aloe for treating burns and found that there was insufficient evidence and insufficient documentation to approve it as a product. Other studies have shown that aloe accelerated wound healing under normal circumstances or for patients with frostbite (Fulton, 1990, *J. Dermatol. Surg. Oncol.* 16(5):480; McCauly et al., 1990, *Postgrad. Med.* 88(8):67). However, another study found that aloe delayed wound healing (Schmidt and Greenspon, 1991, *Obstet. Gynecol.* 78(1):115).

12.4. FRACTIONAL ANALYSIS

Fractional analysis of the carbohydrate components of aloe is performed using gel filtration chromatography as described above.

12.5 BIOLOGICAL ACTIVITY ANALYSIS

Aloe has been shown to have many biological effects. It is known to stimulate peristalsis, inhibit gastric secretion and dilate capillaries. Aloe also has mitogenic activity, hemagglutination activity and antithromboxane activity. The primary assays used are the hepatocyte enzymes, the rat surgical wound healing model, cyclooxygenase-1, cyclooxygenase-2 and lipoxygenase assays. The cyclooxygenase and lipoxygenase activities are performed as in the Saw Palmetto section above. The antithromboxane activities are analyzed as in the ginger section above. Aloe extracts and fractions are analyzed for wound healing ability as described

in U.S. Patent No. 5,487,899, Davis, a rodent wound healing model for analyzing the ability for aloe, either alone or in combination with other agents, to assist in healing of wounds.

12.6. CHEMICAL ANALYSIS

There are many putative components in aloe, including barbaloin, iso-barbaloin, o-glycosides, polysaccharide glucomannan, bradykininase, tannins and magnesium lactate.

Chemical analysis is performed by HPLC and GPC. In addition, the chemical analysis of aloe may also be performed by gas chromatography-mass spectroscopy (GC-MS). A recent example of the chemical analysis for the components of aloe was performed by Yamaguchi in 1993 (Yamaguchi et al., 1993, *Biosci. Biotech. Biochem.* 57(8):1350-1352). There they studied hexane extracts and acetone extracts of freeze-dried aloe.

The gel primarily consists of several types of polysaccharides, including an acidic galactan, mannan, a glucomannan, an arabinan, and/or a glucogalactomannan. Polysaccharides constitute 0.2-0.3% of the fresh gel. (Tyler & Foster, 1996)

The components include aracedonic acid as a prostaglandin precursor (Afzal et al., 1991, *Planta Med.* 57:38-40). The other major components include polysaccharides, including acidic galactan, mannan, glucomannan, arbinan and glucogalactomannan. Polysaccharides make up 0.2-0.3 of the fresh gel. The polysaccharide content is determined by GPC. The glycomannan isolated from aloe was reported by Mandel and Das, 1980 (Mandel and Das, 1980, *Carbohydrate Research* 87:249-256). Other chemically-active compounds in aloe include lectin-like proteins which show anti-cancer and anti-inflammatory activities (Winters et al., 1981, *Economic Botany* 35(1):89-95; Winters et al., 1995, *Phytotherapy Research* 9:395-400). Other small molecule

components of aloe include various fatty acids and various hydrocarbons (Yamaguchi et al., 1993, *Biosci. Biotech. Biochem.* 57(8):1350-1352).

13. EXAMPLE: BILBERRY, *Vaccinium Myrtillus*

It is typically administered either in the fruit form of berries or as an extract. The extract is typically standardized to 36% anthocynosides in doses of 160 mg, once or twice a day.

13.1 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of bilberry. One of the major proprietary products is Tegens™, by Indena - Inverni della Beffa Research Laboratories (Milan, Italy). Myrtocyan™ (Morazzoni et al., 1990, Fitoterapia, v LXI: 1) is the trade name for an anthocyanoside complex from *V. myrtillus* (one component in Tegens™). Bilberry extracts are also available from Natural Factors Nutritional Products, Ltd. (Burnaby, British Columbia, Canada) and Murdock Madaus Schwabe (Springville, Utah). Bilberry in capsule form, 25% anthrocyanosides, is available through Herbal Choice-Botalia, PhytoPharmica, Source Natural, Nature's Herbs and Nature's Way.

13.2 CLINICAL INDICATIONS

The clinical indications of bilberry are varied. The primary indication is venous insufficiency for the lower limbs, varicose veins, atherosclerosis and degenerative retinal conditions. It is believed that this is due to capillary strengthening activity and improvement of blood vessel contractility. Bilberry has also been shown to have anti-inflammatory activity, anti-diarrhetic activity, wound healing activity, and vasoprotective activity. The botanical having vasoprotective and anti-inflammatory activity may be

administered either topically or intravenously (Lietti et al., 1976, *Arzneimittel-Forschung* 26(5):829).

13.3 FRACTIONAL ANALYSIS

The components of bilberry include tannins, anthrocyanins, flavonoids, various invert sugars and pectins. The components of bilberry are separated by HPLC, GPC or gel filtration. Examples of columns include polymer laboratories PL-gel 5m 500Å polymer, Toyopearl 8HW 40S column, and Sephadex™ LH-20 dextran column is also used. The material subjected to the partitioning is performed with ethyl/acetate-water solution, gel filtration with water and/or alcohol and/or water and/or acetone mobile phase. The HPLC is performed using reversed phase column chromatography, (RP-HPLC) using water and/or water and acetone and/or water and alcohol as the mobile phase. For GPC, water and/or water and alcohol and/or water and acetonitrile with or without buffer are used. On the Sephadex™ column, ethanol or chloroform is used, ethanol as the eluent.

13.4 BIOLOGICAL ACTIVITY ANALYSIS

The primary biological methods of analysis for bilberry for venous insufficiency indication is by a blood vessel contractility assay and platelet aggregation. For the anti-inflammatory activity, the primary assays are cyclooxygenase -1 and -2 and 5-lipoxygenase. The experiment is performed as described in the Saw Palmetto section above.

The platelet activating factor assay is performed as described by Morazzion and Magistretti (1990, *Fitoterapia* 61(1):13-21). Platelet aggregation *in vitro*, platelet aggregation *in vivo* and platelet adhesiveness *ex vivo* are determined. Another assay for the venous insufficiency clinical indication of the vasodilator inhibitory effects is done by the study of contractile response of coronary artery

segments to acetycholine (Bettini et al., 1991, *Fitoterapia* 62(1):15-28).

13.5 CHEMICAL ANALYSIS

The chemical analysis is performed using HPLC. The primary components are: tannins, anthocyanins, flavonoids, plant acids, invert sugars, pectins.

14. EXAMPLE: BLACK COHOSH, *Cimicifuga racemosa*

14.1 PLANT SOURCE/BACKGROUND

Black Cohosh is a drug that is administered as either a dried rhizome or roots. It is a member of the buttercup family. It grows in open woods and at the edges of dense forests from Ontario to Tennessee and as far west as Missouri. Black Cohosh has traditionally been used for the treatment of dysmenorrhea, dyspepsia and rheumatism.

14.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Black Cohosh. One of the major proprietary products is Remifemin™, by Shaper & Brummer (Salzgitter, Germany). Another European product is called Klimadynon™ (Jarry et al., 1995, Phytopharmaka Forsch. Klin. Anwend. - Conference Proceedings pp. 99-112). Also, a product sold simply as Wild Appalachian Black Cohosh Root Extract™ is available from Murdock Madaus Schwabe (Springville, Utah).

14.3 CLINICAL INDICATIONS

The German Commission E Monograph describes the use for premenstrual discomfort and dysmenorrhea. It is typically administered in daily doses of 40-200 mg either as a tea, a decoction or as a tincture in alcohol. The clinical indications for Black Cohosh are premenstrual syndrome,

dysmenorrhea, dyspepsia, rheumatism, sore throat, bronchitis, sedative and as a menstrual cycle regulator. In one clinical study the ethanol extract was found to cause selective reduction of the serum concentrations of pituitary luteinizing hormone (LH) (Bradley ed. British Herbal Compendium, Vol. 1, British Herbal Medicine Association:Dorset, England, 1992).

14.4. FRACTIONAL ANALYSIS

Clinical analysis of Black Cohosh is performed using reverse phase HPLC as described in the St. John's Wort section above.

14.5. BIOLOGICAL ACTIVITY ANALYSIS

The primary bioassays for the premenstrual syndrome clinical indication are the estrogen receptor binding assay, luteinizing hormone assay, the FSH bioassay and the oxytocin bioassay.

14.5.1. ESTROGEN RECEPTOR

The *in vitro* binding of the fractions of the total material and the components of Black Cohosh is assayed by several estrogen assays. One assay is the binding of two frozen calf uterus estrogen receptors. The second assay is estrogenic antagonism *in vitro*. A third assay is a rat uteri estrogen receptor model.

Black Cohash extract and fractions are administered p.o. (10 mg/kg) for three consecutive days to a group of 5 ICR derived immature female mice weighing 13 ± 1 g and challenged with estradiol benzoate (3 μg/kg s.c.) immediately after the final dose. The animals are sacrificed 24 hours after the final dose and wet weight of the uterus of each animal is measured. A 50 % or more (≥50) reduction in the estradiol-induced increase in uterine weight indicates estrogen

antagonist activity. (Hirotsu et al., 1986, *Arzneim.-Forsch.* 38 (11): 1410-1416).

Compound	ED50 mg/kg p.o.
*Tamoxifen	0.1 x 3

*Indicates standard reference agent used.

The binding of the total Black Cohosh extract, the Black Cohosh fractions and the components to the rat estrogen receptor is performed as described in Jarry et al., 1985 (Jarry et al., 1985, *Planta Med.* 4:316-319).

14.5.2 THE OXYTOCIN RECEPTOR AGONIST ASSAY

A strip of the uterus obtained from Long Evans derived female rats weighing 275 ± 25 g and sacrificed by cervical dislocation is used. The tissue is placed under 1 g tension in a 10 ml bath containing phosphate buffered saline (pH 7.4) at 32°C. Black Cohash and fractions are introduced at 30 μM. The induced isometrically recorded contraction by more than 50% within 5 min, relative to control 2 nM oxytocin response, indicates possible oxytocin receptor agonist activity. At a test substance concentration where no significant agonist activity is seen, ability to reduce the oxytocin-induced contractile response by more than 50% indicates oxytocin receptor antagonist activity. (Pettibone et al., 1991, *J. Pharmacol. Exp. Ther.* 256: 304-308).

Agonist (#45900)	EC_{50} (μM)
*Oxytocin	0.0012
Prostaglandin $F_2\alpha$	0.035

Antagonist (#45901)	EC_{50} (μM)
*d$(CH_2)_5$[Tyr(Me)$_2$, Thr$_4$, Orn$_8$]OT$_{1-8}$	0.012

*Indicates standard reference agent used.

14.5.3 THE LH REDUCTION ASSAY

The luteinizing hormone assay (LH) is performed as described by Jarry and Harnischfeger, 1985 (Jarry and Harnischfeger, 1985, *Planta Med.* 1:46-49).

14.6. CHEMICAL ANALYSIS

The components in Black Cohosh include the steroidal triterpenes (actein, cimigoside, 27-deoxyactein, cimicifugin, tannin and the alkaloids). Isoflavones are also present in Black Cohosh, including forononetins. Minor constituents also include isoferulics and salicyclics. They are analyzed by HPLC.

15. EXAMPLE: CHAMOMILE, *Matricharia recutita*

15.1 PLANT SOURCE/BACKGROUND

The drug chamomile consists of the dried flower head of German chamomile, *Metricaria recutita*. There is also Roman chamomile obtained from *Chamaelum noble* which is different than German chamomile. Use of Roman chamomile is largely in the United Kingdom. The German therapeutic monograph also has chamomile preparations for use for gastrointestinal spasms and gastrointestinal tract inflammatory diseases. Topical products are also used.

15.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Chamomile. One of the major proprietary products is Kamillosan™, by Asta Medica AG (Frankfurt am Main, Germany). Chamomile dry extract is available from Indena s.a. (Milan, Italy) and is standardized to contain 1% total apigenin and 0.5% essential oil. Also, a product sold simply as German Chamomile Flowers™ is available from Murdock Madaus Schwabe (Springville, Utah).

15.3 CLINICAL INDICATIONS

Chamomile has many clinical indications. Primary indications are use as an anti-spasmodic for GI distress and dermatological anti-inflammatory agent. Other indications include treating the common cold, dyspeptic complaints, acne, inflammation of mouth and wound healing. Chamomile is also reported to have bactericidal and fungicidal activities. Typically, infusion of one or two grams of the flowers or 1 to 4 millimeters of tincture are used for gastrointestinal spasms. Traditional uses include treating cholic, diarrhea, indigestion, insomnia, infantile convulsions, toothache, and bleeding and swollen gums. One example of the anti-microbial use of chamomile is as a vaginal douch as described in U.S. Patent No. 5,244,885, Carle et al.

15.4 FRACTIONAL ANALYSIS

Fractional analysis for chamomile is performed in a manner similar to that described above for St. John's Wort using reverse phase chromatography.

15.5 BIOLOGICAL ACTIVITY ANALYSIS

There are many ways to perform the biological analysis of chamomile, including enzymatic assays, tissue culture assays and co-organism assays. The enzymatic assays for the anti-inflammatory activity are cyclooxygenase-1 and -2 and 5-lipoxygenase. These assays are performed as described in the Saw Palmetto section above.

The muscle spasm tissue culture assay is performed on isolated guinea pig ileum. The spasmodic assay is performed as described in Carle and Gomaa, 1992 (Carle and Gomaa, 1992, *Drugs of Today* 28(8):559-565; Achterrath-Tuckermann et al., 1980, *Planta Med.* 39:38). Whole animal assays are also performed, including the Ca^{2+} influx to mast cells and the mouse ear dermatitis and rat paw swelling assay. The mouse ear experiment is as described below.

15.5.1 MOUSE EAR DERMATITIS MODEL

Chamomile extract, oil and fractions are administered i.p. (0.1, 1, 10 and 100 mg/kg) to groups of 5 ICR derived male mice weighing 22 ± 2 g one hour before sensitization to oxazolone (0.1 ml of 5% solution) applied to the preshaven abdominal surface. Seven days later, animals are challenged with oxazolone (25 µl of a 2% solution) applied to the right ear, vehicle being applied to the left ear. After 24 hours, each mouse is sacrificed and ear thickness measured with a Dyer Model micrometer gauge. A 30 % or more (≥30) enhancement relative to the vehicle treated control group is considered significant and indicates possible immunostimulant activity. (Griswold et al., 1974, *Cell. Immunol.* 11: 198-204).

Compound	ED_{30} mg/kg i.p.
*Azimexone	100
Bestatin	1
Levamisole	30

*Indicates standard reference agent used.

15.5.2 THE RAT PAW SWELLING ASSAY

The paw inflammation experiment is performed as described below. Chamomile extract, oil and fractions are administered p.o. (100 mg/kg) to a group of 3 Long Evans male or female overnight fasted rats weighing 150 ± 20 g one hour before right hind paw injection of carageenan (0.1 ml of 1% suspension intraplantar). Reduction of hind paw edema by 30 % or more (≥30) three hours after carageenan administration indicates significant acute anti-inflammatory activity. (Winter et al., 1962, *Proc. Soc. Exper. Biol. Med.* 111: 544-547)).

Compound	ED_{30} mg/kg p.o.
Acetazolamide	>50
Aspirin	150
BW 755C	30
Clonidine	1
Diflunisal	30
Furosemide	50
Hydrocortisone	30
Hydroflumethiazide	>50
Ibuprofen	30
*Indomethacin	3
Ketoprofen	3
Naproxen	3
Phenidone	50
Phenylbutazone	50
Probenecid	>50
Salbutamol	10

*Indicates standard reference agent used

BW 755C=3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline.

15.6 CHEMICAL ANALYSIS

The chemical analysis is performed by HPLC.

The components include alpha-bisabolol, bisaboloxide a and b, and chamazulene. The components also include flavonoids such as epigenin, leutolin, percitin, myrricitin, epigenin 7 glucoside and erutin.

16. CHASTE TREE, *Vitex agnus-castus*.

16.1 PLANT SOURCE/BACKGROUND

The source of the chaste tree berry is the fruits of *vitax agnus cultus l.* shrub, a small tree native to West Asia and southwestern Europe and also widely distributed in North America. The chaste tree has been used for over 2,000 years for menstrual problems and is typically administered as

either an alcoholic extract of the powdered fruit for a dose of 20 mgs of the dried fruit or administered as a decoction using 30-40 mgs of fruit in a daily dose.

16.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of agnus castus. Some of the products are: Femaprin™, by Murdock Madaus Schwabe (Springville, Utah); Agnolyt™, by Madaus A.G. (Koln, Germany); and Strotan™ capsules (Hamburg, Germany). Extracts are also available from Bionorica™ (Germany). Femaprin-Vitex™ in capsule form, agnuside, 0.15% is available through Nature's Way.

16.3 CLINICAL INDICATIONS

The clinical indications for the chaste tree are many, including attenuating PMS syndromes and menstrual cycle regulation, anti-infective activity and increased lactation. It is also known to assist in mastalgia and menopausal symptoms, and treat inadequate lactation. It is also used as an anti-spasmodic.

16.4 FRACTIONAL ANALYSIS

Fractional analysis of the chaste tree is performed in a manner similar to that of the Saw Palmetto described above. Fractionation is performed on silica gel chromatography beginning in hexane and working toward more polar solvents such as ethyl acetate. Alternatively, reverse phase RP chromatography is done using a C-18 column and working from acetonitrile to water as the solvent systems.

16.5 BIOLOGICAL ACTIVITY ANALYSIS

Biological analysis of chaste tree extract, chaste tree berries, chaste tree fractions and specific compounds from the chaste tree is based on using the following assays: analyzing for dopamine antagonistic activity, measuring the inhibition of prolactin secretion, increasing luteinizing hormone (LH), decreasing follicle stimulating hormone. Chaste tree extracts also have been shown to have anti-microbial activity.

The primary assay is the inhibition of prolactin secretion from rat pituitary cells. (Sliutz et al., 1993, *Horm. Motab. Res.* 25:253-255). In this assay, a cell culture system using freshly harvested rat pituitaries is assayed for release of prolactin with a positive control of thyroxin releasing hormone (TRH). Other workers have found it active in an assay.

The luteinizing hormone (LH) assay is performed as described above in the Black Cohosh example (Jarry et al., 1985).

16.6 CHEMICAL ANALYSIS

Chemical analysis is performed by GC-MS or HPLC. The GC-MS analysis of the chaste tree components is done using published procedures (Zwaving and Bos, 1996, *Planta Med.* 62:83-84).

The components of chaste tree include limonene, cineole, sabinene, flavonoids, brantin and isovitexin.

17. EXAMPLE: CHESTNUT - HORSE, *Castanea dentata, Aesculus hippocastanum*

17.1 PLANT SOURCE/BACKGROUND

Horse Chestnut is the fruit of the tree *aesculus*, which grows to be 75 feet tall and is common throughout the United States and Europe. When the husk dries, the nuts are

released. Extracts of the Horse Chestnut are commercially available for a variety of uses, including varicose veins and hemorrhoids.

17.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Horse Chestnut. One of the major proprietary products is Venostasin retard™, by Klinge Pharma (Munich, Germany). A liquid herbal extract of the fresh mature seeds of the Horse Chestnut is available from Herb Pharm (Williams, Oregon) - this extract contains 58-64% grain alcohol and distilled water. Horse Chestnut dry extract is available from Indena s.a. (Milan, Italy) and is standardized to contain 20% triterpene saponins calculated as aescin. Also, a powdered extract is available (min. 20% aescin) from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany).

17.3 CLINICAL INDICATIONS

The clinical indications for Horse Chestnut are as follows: venous insufficiency, hemorrhoids, varicose veins, eczema, menstrual pain and phlebitis. One clinical study on 22 patients using the commercially available Horse Chestnut extract Venostatin™. They found Venostatin™ had an inhibitory effect on edema formation by decreasing transcapillary filtration (Bisler et al., 1986, *Dtsh. Med. Wochenschr.* 111(35):1321-1329).

Another study reported increased gonadotropin release in menopausal women. Düker et al. reported in 1990 that Remifemin™, an ethanolic extract of Horse Chestnut, reduced luteinizing hormone (LH) secretion. In that study, LH levels, but not FSH levels, were significantly reduced in patients receiving the extract. Extract of Horse Chestnut has been particularly effective in patients with chronic venous insufficiency (Hitzenberger, 1989, *Wien. Med. Wochenschr.*, 139(17):1385; Diehm et al., 1992, *Vasa* 21:188).

17.4 FRACTIONAL ANALYSIS

There are several ways to perform the fractional analysis of the Horse Chestnut extract. Silica get chromatography is performed with solvents such as acetone and working toward a more potent solvent such as methanol. Alternatively, reverse phase chromatography may be performed using acetonitrile and water as the solvent system. Others have reported separation of components on a Sephadex™ LH-20 column using methanol as the eluent (Düker et al., 1991, *Planta Med.* 57:420-424)).

17.5 BIOLOGICAL ACTIVITY ANALYSIS

The components of the Horse Chestnut extract, the fractions and the specific chemical markers are analyzed in a variety of fashions, including the spontaneously activated rat portal vein model, the isolated dog vein model, and lymphatic edema in rats. The anti-inflammatory activity of the Horse Chestnut extract fractions in specific compounds is measured by the carageenan edema induced rat model and the anti-inflammatory assays discussed above, namely, the cyclooxygenase-1 and cyclooxygenase-2 and the 5-lipogenase models.

The portal model is performed using the following procedures:

17.5.1 PORTAL VEIN, SPONTANEOUSLY ACTIVATED

A portal vein ring obtained from Long Evans derived male or female rats weighing 275 ± 25 g and sacrificed by cervical dislocation is used. The tissue is placed under 2 g tension in a 10 ml bath containing phosphate buffered saline (pH 7.4) at 32°C and permitted to develop spontaneous myogenic contractions. When the Horse Chesnut powder, seeds or fraction at 30 μM induces isometrically recorded relaxation by more than 50% within 10 min it indicates significant

activity (Hamilton et al., 1986, *Br. J. Pharmacol.* 88: 103-111).

Agonist	EC_{50} (μM)
Cromokalim	0.17
*Pinacidil	0.84

*Indicates standard reference agent used.

The dog vein model is performed using published methods (Guillaume and Padioleau, 1994, *Arzneimittleforschung* 44:25-35).

The anti-inflammatory activity of steroidal constituents of the Horse Chestnut is analyzed using the estrogen receptor assay which measures modulation of the binding of [^{3}H]estradiol to estrogen receptors. Cytosol from frozen calf uterus is prepared in modified Tris-HCl pH 7.4 buffer using standard techniques. A 100 μg aliquot of cytosol protein is incubated with 1.5 nM [^{3}H]estradiol for 14-16 hours at 4°C. Non-specific binding is estimated in the presence of 5.8 μM diethylstilbestrol. Bound [^{3}H]estradiol is separated from free radioligand by adsorbtion to dextran-coated charcoal. After low speed centrifugation an aliquot is removed from the supernatant and counted to determine [^{3}H]estradiol specifically bound. Samples of Horse Chestnut are screened at 10 μM, based on an assumed average molecular weight of 200. (McGuire et al. eds., Estrogen Receptors in Human Breast Cancer. Raven Press, N.Y. 1975).

Assay Reference Data:

K^d 0.06 nM

B^{max}: 42 fmol/mg protein

Specific Binding: 75%

Compound	IC50 (nM)	K^i (nM)	nH
*Diethylstilbestrol	0.65	0.025	1.0
17-α-Estradiol	1.1	0.041	0.7
Ethynylestradiol	0.081	0.003	1.1

*Indicates standard reference agent used.

Analysis is performed using the rat paw edema model following the protocol described above in the Chamomile section. Alternatively, published protocols are used (Düker, 1991, *Planta Med.* 57:420-424; Senatore, 1989, *Boll. Soc. It. Biol. Sper.* 65(2):137-141).

17.6 CHEMICAL ANALYSIS

Chemical analysis is performed using HPLC. The components include deic acids, phenolic acids, coumarins, cyclitols, aesain, saponins, various triterpens, various flavonoids, polypreinoids, monoterpines, lipids and carbohydrates.

18. EXAMPLE: ECHINACEA, *ECHINACEA ANGUSTIFOLIA* AND PURPUREA

18.1 PLANT SOURCE/BACKGROUND

Echinacea is typically administered in a dose of 900 mg per day. It is often administered in the form of a tincture (1:5) prepared with 50% ethanol. Echinacea is administered in the form of tablets or capsules and there the dose is 1 g three times daily. Echinacea has been approved by the German Commission E Monograph (B Anz. No. 162, dated August 29, 1992).

Echinacea angustifolia, often called purple cornflower, is a plant of North American origin. Native Americans have used extracts from this plant for wound healing (antibiotic) and as an anti-inflammatory agent. Freshly squeezed juices

of leaves and roots from this plant have been approved by the German government for the treatment of recurrent infections of respiratory and urinary tracts. Liquid Echinacea preparations have been shown to have immune stimulatory activity when administered orally or parenterally. It is believed that the activation of splenocytes may contribute to the extract's ability to enhance the activity of granulocytes and phagocytes.

For fingerprinting in accordance with the present invention, green leaves and roots are cut and frozen at less than -100°C. The mixture is then pulverized and extracted with a known volume of water. This procedure is preferred to retain the maximum amounts of components. The components are a mix of volatile oils, glycosides, amides and polyacetylenes.

18.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

Echinacea is one of the most popular botanical products available.

One of the major proprietary products is Echinacin™, by Madaus AG (Koln, Germany), and a similar product is sold in the U.S. as Echinaguard™, by Murdock Madaus Schwabe (Springville, Utah).

Also, a powdered extract of *Echinacea angustifolia* is available (min. 4-5% echinosides) from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany). Other suppliers include Trout Lake Farm, PhytoPharmica, Herbal Choice-Boatalia, Shaklee, Botalia Gold, Nature's Herbs, Nature's Way, Flora Laboratories, and Herb Pharm.

18.3 CLINICAL INDICATIONS

The clinical indications of Echinacea are many. The primary indication is increasing resistance to infection, colds, etc., in the upper respiratory tract. Other indications are superficial wound healing, treatment of recurring urinary tract infections or respiratory tract infections, herpes simplex, anti-inflammatory activity and anti-bacterial activity.

18.4 FRACTIONAL ANALYSIS

The fractional analysis is performed as described in the mistletoe experiments above using Sephadex™ chromatography. Alternatively, reverse phase C-18 chromatography or GPC chromatography may also be used. There are two major categories of bioactive components in Echinacea, the non-polar material that is extracted with chloroform and the polar component which is in the ethanol or aqueous fraction. The non-polar lipophilic constituents include various anions as described by Bauer and Foster (Bauer and Foster, 1991, *Planta Med.* 57:447-449). The polar components include the alkamides and the polysaccharides (Bauer and Remiger, 1989, *Planta Med.* 55:367-371; Steinmuller et al., 1993, *J. Immunopharmacol.* 15(5):605-614).

18.5 BIOLOGICAL ACTIVITY ANALYSIS

The bioactivity of the Echinacea total extract and fractions are analyzed in an assay which measures the modulation of the binding of [^{125}I]tumor necrosis factor-α (TNF-α) to human TNF-α receptors. U-937 (human histiocytic lymphoma) cells are used to prepare membranes in modified Tris HCl buffer (pH 8.6) using standard techniques. A 200 μg aliquot is incubated with 62 pM [^{125}I]TNF-α for 3 hours at 4°C. Non-specific binding is estimated in the presence of 50 nM TNF-α. Membranes are filtered and washed 3 times and the filters are counted to determine [^{125}I]TNF-α specifically

bound. Compounds are screened at 10 μM. (Maloff and Delmendo, 1991, *Agents and Actions* 34: 32-34).

Assay Reference Data:

K^d 37 pM

B^{max}: 11 pM mg protein

Specific Binding: 65%

Compound	IC_{50} (nM)	Ki (nM	nH
Interleukin-1α (IL-1α)	>500	-	-
*TNF-α	0.084	0.032	1.2
TNF-β	0.714	0.27	1.0

*Indicates standard reference agent used.

Another clinical indication of Echinacea is anti-inflammatory activity. The anti-inflammatory activity may be analyzed using a variety of methods. Three *in vitro* assays that are used include the cyclooxygenase-1, cyclooxygenase-2 and lipoxygenase assay, described in the Saw Palmetto section above. Alternatively, arachidonate metabolites may be analyzed following the procedure of Wagner et al., 1989, (*Planta Med.* 55:566-567). Alternatively, *in vivo* assays such as the rat paw assay or the mouse ear assay are performed as described in the Chamomile section or as in literature preparations (Tragni et al., 1985, *Chem. Toxic.* 23(2):317-319).

The extract is separated into basic classes of components following the procedure set forth in FIG. 3. Each separated class of components is subjected to two types of bioassays (as well as the original extract). The first assay measures the activation of spleen cells in C57BL/6 mice. The mice are sensitized with P815Y lymphoma and treated with different doses of the sterile extract or specific components. After 11 days post lymphoma cell inoculation, the spleens are harvested and splenocytes used to challenge

P815Y lymphoma cells loaded with ^{51}Cr. The release of the radioactive ^{51}Cr from tumor cells provides a measure (radioactive counts) of the activation of splenocytes (the assay system).

A bioassay for a secondary clinical indication, antibacterial activity, is performed in cultures of *E. coli* using the turbidity of growing *E. coli* as a measure of their growth. Lack of turbidity in the presence of a given test extract or its components provides an indication of antibacterial activity.

18.5.1 BIOASSAY FOR MACROPHAGE ACTIVATION

Echinacea extract and its polysaccharides can stimulate macrophage activity. In experimental systems, macrophages can be obtained by i.p. injection of mice with 2-3 ml. of 2% starch or thioglycolate. After 4-5 days peritoneal cells of the mice are collected with i.p. injection of 5 ml. PBS. The buffer is removed with a syringe and the harvested cells are collected by centrifugation and incubated at 37°C in RPMI 1640 medium containing 10% fetal calf serum. After one (1) hour, non-adherent cells are discarded and the remainder used for testing. The macrophages are treated with different concentrations of Echinacea extract and the Echinacea fractions in the culture media and activation qualified by the cytotoxicity against ^{51}Cr loaded P815 cells as targets (Stimpel et al., Infection and Immunity, 46:845-849).

18.6 CHEMICAL ANALYSIS HPLC, GPC

The most bioactivity associated components (*e.g.* terpenoids) are separated by GC, GC/MS or HPLC technology into individual components which are used to provide fingerprints in accordance with the present invention.

In hydroalcoholic preps the following compounds are found: echinacosides, arabinogalactan, heteroxylan (activity potentiated by isolutylamides, chicoric acid). The detailed

analysis of the chemically-active components of Echinacea is performed using published procedures (Bauer and Foster, 1991; Bauer and Reminger, 1989; Bauer et al., 1989, *Zeit. fur Phytotherapie* 10:43-48).

19. EXAMPLE: EVENING PRIMOSE, *OENOTHERA BLENNIS*

19.1 PLANT SOURCE/BACKGROUND

The drug evening primrose is the oil expressed from the seeds. It is native to North America, but widely naturalized everywhere, and a member of the primrose family. The seeds have about 14% oil content of which the oil is cis linoleic acid (50-70%). The next most prevalent component is gamma-linoleic acid (7-10%). The primary active component is thought to be gamma-linoleic acid (GLA). The dose for GLA supplementation conditions is 600-6,000 mg per day for atopic eczema. The dosage is 250 mg capsules taken twice daily.

19.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of evening primrose oil. One well known product is called *Efamol*™ or Evening Primrose Oil™, by Efamol Research Inc., a subsidiary of Scotia Holdings (Kentville, Nova Scotia, Canada). Another product is known as Health From the Sun Evening Primrose Oil™ capsules, by PGE Canada (Saskatoon, Saskatchewan, Canada) - which are made with a "hexane-free extraction process". Additional EPO products are: Epogam™ and Gammaoil™. (Morse et al., 1989, *Br. J. of Dermatology*, 121: 75-90), Royal Brittany Evening Primrose Oil™, available through American Health.

19.3 CLINICAL INDICATIONS

The clinical indications for using evening primrose are many, including cardiovascular disease, cancer, rheumatoid arthritis, premenstrual syndrome, multiple sclerosis, atopic eczema and other topical diseases. Minor indications include

diabetes, hepatoprotectant for alcoholics, autoimmune diseases, childhood hyperactivity, treating chronic inflammation, treating ethanol-induced toxicity, treating acute alcohol withdrawal syndrome, ichthyosis vulgaris, scleroderma, Sjogren's syndrome, Sicca syndrome, brittle nails, mastalgia, psychiatric syndromes, tardive dyskinesia, ulcerative colitis, migraine headaches, and liver cancer. The primary clinical indication of interest is the treatment of eczema (Bordoni et al., 1987, *Drugs Exptl. Clin. Res.* 14(4):291-297). The effectiveness against eczema is believed to be due to GLA. Prostaglandins, particularly PGE_1, seem to be involved in the regulation of the T lymphocytes and patients with atopic eczema typically have a low PGE_1 level.

Other workers have reported that evening primrose oil is effective in reducing mastagyna or breast pain (Gateley and Mansel, 1991, *British Medical Bulletin* 47(2):284-294). In addition, evening primrose oil has been reported to be effective in experimental models for diabetes (Stevens et al., 1993, *Prostaglandins, Leucotrins and Essential Fatty Acids* 49:699-706). Lastly, evening primrose oil has been reported to act as an immunosuppressive agent (U.S. Patent Nos. 3,993,775 and 4,058,594, both to Williams).

19.4 FRACTIONAL ANALYSIS

The fractional analysis for evening primrose is performed by flash chromatography as described in the Saw Palmetto experiments above.

19.5 BIOLOGICAL ACTIVITY ANALYSIS

Biological analysis is performed in two manners. The first method is studying platelet activating factor binding. The second method is to study platelet aggregation using blood from rabbits. The analysis is performed as described below.

19.5.1 PLATELET ACTIVATING FACTOR, PLATELET AGGREGATION

Venous blood obtained from male or female New Zealand derived albino rabbits weighing 2.5-3 kg is mixed with one-tenth volume of trisodium citrate (0.13 M) and centrifuged at room temperature for 10 min at 220 g. Evening primrose oil and fractions at (30 μM) are analyzed for induced aggregation of the supernatant platelet rich plasma by more than 50% within 5 min, relative to 5 nM platelet activating factor-acether (PAF-acether) at 37°C as measured by an optical aggregometer; induced aggregation of >50% , indicates possible PAF receptor agonist activity. At sample concentrations where no significant agonist activity is seen, ability to reduce the PAF-acether-induced maximum non-reversible aggregation response by more than 50% indicates PAF receptor antagonist activity (Nunez et al., 1986, *Eur. J. Pharmacol.* 123: 197-205).

Agonist (#46300)	EC_{50} (μM)
*PAF (Platelet Activating Factor)	0.0018

Antagonist (#46301)	EC_{50} (μM)
Nectandrin A (BN-52021)	3.3
CGS-12970	26
CV-3988	10
Kadsurenone (L-651108)	1.7
L-652731	0.83
L-659989	0.33
RP-48740	17
SRI-63441	1.7
*WEB-2086	0.11

*Indicates standard reference agent used.

CGS-12970=3-methyl-2-(3-pyridinyl)-1H-indole-1-octanoic acid;
CV-3988=3-(4-hydroxy-7-methoxy-10-0x0-3,5,9-trioxa-11-aza-4-

phosphanonacos-1-yl)-thiazolinium; L-652731=2R,5R-di(3,4,5-trimethoxyphenyl)-tetrahydrofuran; L-659989=pyro-2-aminoadipyl-histidyl-thiazolidine-4-carboxamide; WEB-2086=3-(4-[2-chlorophenyl]-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo-[4,3-a][1,4]-diazepine-2-yl)1-(4-morpholinyl)-1-propanone.

19.5.2 PLATELET ACTIVATING FACTOR

In this assay the evening primrose oil and fractions are analyzed by measuring the binding of [^{3}H]platelet activating factor (PAF) to PAF receptors. Platelets of male or female New Zealand derived albino rabbits weighing 2.5-3 kg are prepared in modified Tris-HCl pH 7.5 buffer using standard techniques. A 50 μg aliquot of membrane is incubated with 0.4 nM [^{3}H]PAF for 60 minutes at 25°C. Non-specific binding is estimated in the presence of 1 μM PAF. Membranes are filtered and washed 3 times and the filters are counted to determine [^{3}H]PAF specifically bound. Samples are screened at 10 μM. (Hwang et al., 1983, *Biochemistry* 22: 4756-4763).

Assay Reference Data:

K^d	0.73 nM
B^{max}:	4.6 pmol/mg protein
Specific Binding:	93%

Compound	IC_{50} (nM)	Ki (nM)	nH
*PAF	9	5.8	1.0
WEB-2086	110	71	0.8

*Indicates standard reference agent used.

WEB-2086=3-(4-[2-chlorophenyl]-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]-triazolo-[3,3-*a*][1,4]-diazepine-2-yl)1-(4-morpholinyl)-1-propanone

The immune activity of the evening primrose oil may be studied using the prostanoid FT assay as described in the aloe section above.

19.6 CHEMICAL ANALYSIS

Chemical analysis is performed using HPLC for the linoleic acid and other essential fatty acids (Cisowski et al., 1993, *Phytoterapia* 64(2):155-162). The chemicals are also analyzed as described in the Saw Palmetto experiment above.

Essential fatty acids, gamma linoleic acid, cis linoleic acid are the primary components.

20. EXAMPLE: FEVERFEW, *Tanacetum parthenium*

20.1 PLANT SOURCE/BACKGROUND

Feverfew is a member of the Aster family. It is native to Europe but now naturalized both in North and South America. The herb is typically administered in the form of the dried leaves in tablet or capsule form. A typical average daily dose is 125 mg of leaves having a minimum content of 0.2% parthenolid.

20.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Feverfew. One of the major proprietary products in the U.S. is known as Mygrafew™, by Murdock Madaus Schwabe (Springville, Utah). Also, a powdered extract of Feverfew herb is available from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany) as well as from Natural Factors Nutritional Products, Ltd. (Burnaby, British Columbia, Canada). Herbal Choice-Botalia, Herb Pharm, Nature's Way, Herbal Harvest, Botalia Gold and Herbal Laboratories also supply Feverfew.

20.3 CLINICAL INDICATIONS

The clinical indications for Feverfew are: migraine (topical), exema, relief of menstrual pain, asthma, arthritis, circulatory complaints, sight problems, weariness and sensitivity to light. The primary indication for which it has been approved in France and Canada is treatment of migraine. The Canadian authorities have approved labeling with the claim "used as a prophylactic against migraines." The Canadian product is required to contain 0.2% parthenolid (Awang, 1987, *The Pharmaceutical Journal*, 239:487).

Feverfew has also been the subject of clinical studies for arthritis (Pattrick et al., 1989, *Annals of Rheumatic Disease*, 48:547-549). This study has found that there were no important differences between the control group and those receiving Feverfew but all of the patients had "inadequately controlled inflammatory joint syndromes" and were not responding well before the study started. The authors also note possible benefits of Feverfew in osteoarthritis or soft tissue lesions. An alcoholic tincture of Feverfew may be used topically for eczema.

20.4 FRACTIONAL ANALYSIS

The fractional analysis of Feverfew extracts is performed using reverse phase chromatography as described above in the St. John's Wort section.

20.5 BIOLOGICAL ACTIVITY ANALYSIS

A biological analysis of Feverfew is based on the noted inhibition of prostaglandins synthesis, the prevention of arachidonic acid formation, the inhibition of *in vitro* aggregation of platelets, and serotonin $5HT_1$ inhibition. The serotonin assay is performed as described above in the St. John's Wort section. The platelet aggregation assays are performed as described above in the Evening Primrose section.

Extracts of Feverfew may also be analyzed studying granule secretion in blood platelets and polymorphonuclear leucocytes. Studying both chloroform and methanol extracts and aqueous buffer extracts, Feverfew consistently inhibited platelet aggregation but not thromboxane synthesis (Heptinstal et al., May 11, 1985, Lancet 1071-1073).

20.6 CHEMICAL ANALYSIS

The chemical analysis is performed using HPCL as described in the St. John's Wort section above.

The primary chemical components of Feverfew are sesquiterpenoids, particularly, parthenolid (0.12-1.27%). The leaves contain sesquiterpenoids: artecanin, canin, chrysanthemolide, chrysanthemonin, 10-epi-canin, 1β-hydroxyarbusculin, 8β-hydroxyreynosin, 3β-hydroxyparthenolide, magnoliolide, parthenolide (up to 85% of sesquiterpene content), reynosin, santamarin, seco-tanapartholide A, tanaparthin, tanaparthin-1α, 4α-epoxide, tanaparthin-1β, 4β-epoxide(Leung & Foster, 1996).

21. EXAMPLE: GARLIC, *Allium sativum*

21.1 PLANT SOURCE/BACKGROUND

Garlic as either the dried or the fresh bulbs of *Allium sativm* of the lily family has been grown throughout the world and has been cultivated for more than 5,000 years. An essential oil is also available from garlic.

21.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercia suppliers of garlic. Some of the major products are Kyolic™ garlic capsules, by Wakanuga Pharmaceutical Co., Ltd. (Osaka, Japan - subsidiary in Mission Viejo, Ca), and Garlinase 4000™ by Enzymatic Therapy (Green Bay, Wisconsin). Garlic powder tablets are also available from Lichtwer Pharma, GmbH (Germany) and

garlic capsules are available from Natural Factors Nutritional Products, Ltd. (Burnaby, British Columbia, Canada). Shaklee, Herbal Choice-Botalia, Nature's Way, Sunsource, PhytoPharmica, Lichtwer, Bayer Consumer also supply garlic.

21.3 CLINICAL INDICATIONS

Garlic has many clinical activities. Garlic has many activities including antibacterial, antifungal, antithrombonic and hypotensive activity. It also activates fibrinolysis and is an anti-inflammatory.

Garlic has also been studied for the treatment of high blood pressure, atherosclerosis, hypoglycemia, digestive ailments, colds, flu, bronchitis and blood cholesterol and triglyceride lowering activity (Foster, 1991, "Garlic: Alium Sativum," Botanical Series No. 311 American Botanical Counsel: Austin Texas, pg. 1-7). The hypoglycemic activity has been studied in a rat model (Kamanna and Chandrasekhar, 1984, *Indian J. Med. Res.* 79:580-583). There it was observed that the hypocholesteremic activity of garlic is only in the essential oil fraction. Others have reported that Garlic reduces thrombocyte aggregation in a clinical study (Kiesewetter et al., 1991, *Int. J. Clin. Pharm.* 29(4):151-155). The patients in that study received 800mg of garlic powder (four tablets at 200mg, Lichtwer Pharma GmbH).

21.4 FRACTIONAL ANALYSIS

The fractional analysis of garlic is performed using supercritical CO_2 chromatography or reverse phase chromatograph.

The chromotography is performed using SFC-FSE™ grade C_2 (Air Products and Chemicals Inc.; Allentown, PA) with a 5% phenymethyl poysiloxane column.

21.5 BIOLOGICAL ACTIVITY ANALYSIS Platelet aggregation, PAF, fibrinolysis, hepatocytes to measure cholesterol secretion

The biological analysis of garlic is performed using the platelet activating factor for assay and the platelet aggregation assay as described above in the Evening Primrose Section.

21.6 CHEMICAL ANALYSIS GC-MS, HPLC

The chemical components in garlic include alliin, ajoenes and various other sulfur containing compounds. The analysis is performed using GC-MS or HPLC. The HPLC analysis is done using published procedures (Iberl et al., 1990, *Planta Med.* 56:320-326). The chemical anaysis of garlic is also performed using supercritical fluid chromotography - mass spectroscopy (Calvey et al., 1994).

Garlic contains 0.1-0.36% (usually ca. 0.2% volatile oil, alliin (S-allyl-L-cysteine sulfoxide), S-methyl-L-cysteine sulfoxide, enzymes (*e.g.*, alliinase, peroxidase, and myrosinase), ajoenes (E,Z-ajoene, E,Z-methylajoene, and dimethylajoene), protein (16.8%, dry weight basis), minerals, vitamins (thiamine, riboflavin, niacin, etc.), lipids, amino acids, and others.

The volatile oil contains allicin (diallyldisulfide-S-oxide; diallyl thiosulfinate), allylpropyl disulfide, diallyl disulfide, and diallyl trisulfide as the major components, with lesser amounts of dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, allylmethyl sulfide, 2,3,4-trithiapentane, and other related sulfur compounds. Other volatile compounds present include citral, geraniol, linalool, and α- and β-phellandrene. Protaglandins A_2 and F_{1a} were recently isolated from a homogenized garlic extract (Leung and Foster, 1996).

22. EXAMPLE: GINKGO, *Ginkgo bilboa*

22.1 PLANT SOURCE/BACKGROUND: ORAL AND INTRAVENOUS FORMS

Ginkgo is based on the dried leaves of the *Ginkgo bilboa* plant, a member of the Ginkgo family. Traditionally Ginkgo has been reported for use as an anti-periodic antiseptic, hemostatic diuretic and tonic.

22.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

Ginkgo preparations are among the most popular botanical products available. Egb 761, by IPSEN Research Laboratories (Paris, France) is a commercial extract sold under the brand names Tebonin™, Tanakan™ and Rokan™, and has been used extensively in various European clinical trials. Another ginkgo extract by IPSEN, LI 1370, is sold under the brand name Kaveri. Ginkgo bilboa dry extract is available from Indena s.a. (Milan, Italy) and is standardized to contain 24% total ginkgoflavonglycosides, 6% ginkgolides and bilobalide. The extract is also available from Natural Factors Nutritional Products, Ltd. (Burnaby, British Columbia, Canada), Murdock Madaus Schwabe (Springville, Utah), and Botanicals International, a division of Zuellig Botanicals, Inc. (Germany), Herbal Choice-Botalia, Thompson Nutritional, Hudson, NaturaLife, Botalia Gold, Nature's Resource, Herb Pharm, PhytoPharmica, Nature's Way, Tebonin™ (Schwabe, Germany), Rokan™ (Intersan, Germany) and PhytoPharmica.

22.3 CLINICAL INDICATIONS

The daily dose for Ginkgo is 120-160mg of standardized leaf extract. It has also been used for situations involving inflammation of mucous membranes, hemorrhoids, nasal congestions, sore gums, sore eyes, wounds, sores, acne, dandruff and ringworm. It has also been used in cancer.

The clinical use of Ginkgo has recently been reviewed (Cleijen and Knipchild, 1992, *Lancet* 340:1136-1139). A

primary indication is increasing blood flow. In particular, increasing cerebral blood flow. Ginkgo has also been reported for tinnitus, peripheral vascular disease, intermittent claudication, reduced functional capacity and vigilance (cerebral circulatory disturbances), retinopathy, memory deficits, dizziness, vertigo. Compositions of ginkgo components and uses have been reported in the patent literature (U.S. Patent No. 5,246,216 Bombardelli et al. reporting the use as an anti-infective, particularly for pneumocystis carinii and U.S. Patent No. 5,202,313, Bombardelli et al. to bilobalide derivatives).

22.4 FRACTIONAL ANALYSIS

Because the chemical components are flavinols, flavones, sesquiterpenes, diterpenes, monoterpenes, ginkgolides and their carbohydrate derivatives, the fractional analysis was performed on a C18 Liquid Chromatography System.

22.5 BIOLOGICAL ACTIVITY ANALYSIS

Ginkgo acts as a free radical scavenger, inhibits platelet activating factor (PAF), causes EEG changes, reduces capillary fragility, and increase blood fluidity.

The bio-activity of the Ginkgo extract and the Ginkgo fractions may be analyzed using the platelet activating factor assays, free radical scavenger assays measuring and increases in blood fluidity.

The Ginkgo fractions, extracts and raw material are analyzed using the platelet activating factor assays as described above in the Evening Primrose section.

22.6. CHEMICAL ANALYSIS

Chemical analysis of Ginkgo is performed using GC-MS and HPLC. The primary components are flavonol and flavone (mostly quercetin and kaempferol) glycosides, bilobalaide

(sesquiterpene), isoginkgetin, ginkolide A, B, C, M & J (diterpene lactone derivatives), 6-hydroxykynurenic acid, shikimic acid, protocatechuic acid, vanillic acid, para-hydroxybenzoic acid, proanthrocyanidines, heterosides, bioflavones, sciopitysin, ginkgetin, bilobetin and ginkgolic acid.

23. EXAMPLE: GINSENG (Asian), *Panax Ginseng*

23.1. PLANT SOURCE/BACKGROUND

Ginseng is a dried root of the Asian Ginseng or the American Ginseng, both members of the Ginseng family. It is rare and wild but it is extensively cultivated in China and Korea.

23.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

Ginseng is arguably the most popular botanical product available around the world. Some of the products are Ginsun™ by Murdock Madaus Schwabe (Springville, Utah), GS-500™ by Enzymatic Therapy (Green Bay, Wisconsin), and Ginseng Softgels™ by Natural Factors Nutritional Products, Ltd. (Burnaby, British Columbia, Canada). Powdered Ginseng extracts are available from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany). Ginseng dry extract IDB is available from Indena s.a. (Milan, Italy) and is standardized to contain 7% ginsenosides. Ginseng is also available through the following companies: Shaklee, Lichtwer, Sunsource, Nature's Resource,Herbal Choice-Botalia, Nature's Way, NaturaLife, Herbal Harvest, Botalia Gold and PhytoPharmica.

23.3 CLINICAL INDICATIONS

Typically, Ginseng is administered in a daily dosage of 1 to 2 g of root in an appropriate formulation. It is has been the subject of approval in the German Commission E.

Monograph No. 11 dated January 17, 1991. Ginseng is a complex mixture of various components including: ginsenosides which have varied effects on cardiovascular and nervous systems (Brekhman and Dardymov, 1969, *Ann. Rev. Pharmacol.* 9:419-430). It shows different activity in the cardiovascular system (Kaku et al., 1975, *Arzneim. Forsch.* 25:539-547).

Ginseng also shows anti-cancer activity. One study reported that it protected cultured cells from ionizing radiation (Benhur and Fulder, 1981, *Am. J. Chinese Med.* 9(1): 48-56). There, they reported the Ginseng's ability to increase radiation resistance in cell culture. Others have reported activity as a radioprotective in mice (Yonezaw, 1976, *J. Radiation Res.* 17:111).

Others have reported a variety of central nervous system effects. (Petkov, 1959, *Arzneim. Forsch.* (*Drug Res.*) 9:305-311). It has been shown to delay the onset of convulsions and prolong phenobarbyl's sleeping time. It also stabilizes sleep and wakefulness cycles in food deprived rats (Lee et al., 1990, *Neurosci. Let.* 111:217).

23.4 FRACTIONAL ANALYSIS

Fractional analysis is performed on a reverse phase (RP) column using a C-18 column used with an acetonitrile water gradient using the methods described above.

23.5 BIOLOGICAL ANALYSIS

The biological analysis of Ginseng for the clinical condition of stress release is performed in an assay studying the activation of PC12 cells. The procedure followed is as described in the literature (Mohri et al., 1992, *Planta Med.* 58:321-323). They report that the lipophilic components of Ginseng are able to activate neuronal cells in their model.

23.6 DEPRESSION, TETRABENAZINE

Ginseng, Ginseng extract and fractions are administered p.o. (30 mg/kg) to a group of 3 ICR derived mice weighing 22 ± 2 g 30 minutes after injection of tetrabenazine methane sulfonate (100 mg/kg i.p.) and body temperature is recorded 60 minutes later. Reduction of tetrabenazine-induced hypothermic response by 50 % or more (≥50) is considered significant and may indicate antidepressant activity. (Gylys et al., 1963, *Annals N.Y. Acad. Sci.* 107: 899-913).

Compound	ED50 mg/kg p.o.
Amitriptyline	3
Amphetamine	1
Bupropion	30
Clonidine	>30
Desipramine	1
Fluoxetine	>30
*Imipramine	3
Mianserin	30
Pargyline	10
Salbutamol	>30
Tranylcypromine	3

*Indicates standard reference agent used.

Alternatively, the anti-stress activity of Ginseng is evaluated in a rat *in vivo* model or a mouse *in vivo* model. Experiments studying Ginseng components in these animal models have been reported in the literature (Nabata et al., 1973, *Japan J. Pharmacol.* 23:29-41). They reported on various behavioral studies on mice and rats including the conditioned avoidance test, the motor coordination test and the pole climbing test.

Other researchers have reported that components of Ginseng bind to the GABA-A and the GABA-B receptors (Kimura et al., 1994, *Gen. Pharmacol.* 25(1):193-199).

23.7 CHEMICAL ANALYSIS HPLC

The chemical analysis of Ginseng is performed by HPLC or TLC. Ginseng contains a variety of substituents including sterols (betasitosterol and betaglucoside), 7 to 9% Ginseng polysaccharides, panaxins A through U, pectin, free sugars, biomins, polyacetylines, polypeptides. The sappinins are called ginsenosides by the Japanese researchers and panaxosides by Russian workers. There are at least 18 sappines found in Asian Ginseng. They are all triterpenoids. Six panaxosides have been reported. Ginseng oil also is reported to contain sesquiterpeneuiturpines and there are at least 56 closely related saponins called gynosaponins (Leung and Foster, 1996).

24. EXAMPLE: GOLDENSEAL, *Hydrastis canadensis*

24.1 PLANT SOURCE/BACKGROUND

Goldenseal is a perennial found in the deep woods from Vermont to Arkansas in the U.S. It is a member of the Buttercup family. Preparations are traditionally used for their anti-microbial, astringent and anti-hemorrhagic activity.

24.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of suppliers of Goldenseal. Some of the products are: Wild American Goldenseal Root™ capsules, by Murdock Madaus Schwabe (Springville, Utah), and Goldenseal Root Powdered Extract™, by Botanicals International, a division of Zuellig Botanicals, Inc. (Germany), which is standardized to contain 5% total alkaloids and 5% hydrastine. Goldenseal extracts standardized to 5% Hydrastine are also available from Indena s.a. (Milan, Italy).

24.3 CLINICAL INDICATIONS

There have been few clinical studies of Goldenseal. However, it does contain a great deal of berberine which is an antibacterial and amoebicidal agent. Particularly, its often used for the treatment of mucosal inflammation. It has been approved in Germany under the preparation named Gingivitol (the content of the approved drug is specified to have hydrastine from 1.5% to not less than 2.5%).

Total dosage is 0.5g of dried root or 2 ml to 4 ml of tincture (1:10, 60% ethanol) 3 times a day (Bradley, 1992).

24.4 FRACTIONAL ANALYSIS

The fractional analysis of Goldenseal is performed using either C-18 reverse phase (RP) chromatography or silica gel chromatography. The conditions are as described above.

24.5 BIOLOGICAL ACTIVITY ANALYSIS MAO-A, SEROTONIN UPTAKE

The biological analysis of Goldenseal for the premenstrual syndrome clinical indication is performed as described in the oxytocin assay described in the Black Cohash section above. Goldenseal may also be analyzed using the MAO-A assay and the serotonin uptake assay described in the St. John's Wort section.

For clinical use as a mouthwash, the anti-infective, anti-bacterial activity may be measured as described previously.

24.6 CHEMICAL ANALYSIS

Goldenseal contains active principles including iosquinoline alkaloids consisting mainly of hydrastine (1.5-4%) and berberine (0.5-6%), with lesser amounts of canadine (tetrahydroberberine), canadaline, 1-α-hydrastine, 5-hydroxytetrahydroberberine, and other related alkaloids also present. Other constituents include meconin, chlorogenic

acid, lipids with 75% unsaturated and 25% saturated fatty acids, resin, starch, sugar, and a small amount of volatile oil (Leung and Foster, 1996). The chemical analysis of berberine is done by TLC or HPLC. The bio-active components include berberine and hydrastine. These components are primarily isoqinoline alkaloids from both the Rhizome and the root.

25. EXAMPLE: GREEN TEA, *Camellia sinensis*

25.1. PLANT SOURCE/BACKGROUND

C. Sinesis Trauma, Green tea is a large tree with evergreen leaves native to Eastern Asia where it is extensively cultivated. The dried leaves of Green tea have been used to prepare beverages for several thousand years. The tea has been used medicinally for centuries and is regarded as a cure for cancer in China.

25.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a number of Green tea suppliers around the world. Green tea powdered extracts are available from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany) and are standardized to contain 15% tannins and 3-10% caffeine. Green tea extracts are also available from Indena s.a. (Milan, Italy) and are standardized to contain 80% polyphenols.

25.3 CLINICAL INDICATIONS

Green tea has many clinical indications including anti-cancer activity, lowering cholesterol activity, platelet aggregation activity and blood thinning activity. Green tea has also been implicated in increasing longevity. A primary indication is anti-cancer activity which has been reported *in vitro* and *in vivo* (Wang and Wu, 1991, *IARC Sci. Publ.* 105:546). Others have reported that Green tea modified

mutagen-induced chromosomal aberrations (Imanishi et al., 1991, *Mutat. Res.* 259(1):79). Green teas has also been implicated in decreased risk of colon cancer and inhibition of small intestine carcinogenisis (Kono et al., 1991, *J. Clin. Epidemiol.* 44(11):1255; Ito and Imaida, 1992, *Teratogenesis Carcineg. Mutagen.* 12(2):79).

Green tea has also been associated with a decrease in total cholesterol levels (Kono et al., 1992, *Prev. Med.* 21(4):526). Lastly, Green tea has been implicated in improving longevity of Japanese women (Sadakata et al., 1992, *Tohoku J. Exp. Med.* 166(4):475). Green tea also has been shown to be preventative of dental caries (Rosen et al., 1984, *J. Dent. Res.* 63(5):658). The blood thinning activity has been described recently in the published literature (Ali et al., 1990, *Prostaglandins, Leukotrienes and Essential Fatty Acids* 40:281-283).

25.4 FRACTIONAL ANALYSIS

The fractional analysis of Green tea is performed using a variety of different methods including differential extraction (International Publication No. WO 96/28178 Bombardelli et al.). Fractional analysis is performed using Sephadex™ LH-20 chromatography using published procedures (Cattell and Nursten, 1976, *Phyto Chemistry* 15:1967-1976; Sagesaka-Mitane et al., 1990, *Chem. Pharm. Bull.* 38(3): 790-793). Alternatively, fractional analysis may also be performed using silica gel or polyacrylamide (Chkhikvishvli, 1985, *Chem. Nat Compounds* 20(5):629-630).

25.5 BIOLOGICAL ACTIVITY ANALYSIS

For the anticancer clinical indication, there are a variety of bio-assays that may be performed both *in vitro* and *in vivo*. A P450 assay is performed (Obermeier et al., 1990, *Xenobiotica* 25(6): 575-584). In that study the workers studied the effects of bioflavonins on hepatic P450

activities in rat and human liver microsomes. Alternatively, the anti-cancer activity *in vitro* is studied by the chemical anti-oxidant activity (Tanizawa et al., 1984 *Chem. Pharm. Bull.* 32(5):2011-2014). Alternatively, the L1210 cancer model described in the mistletoe section above may be used.

25.6 CHEMICAL ANALYSIS

The chemical analysis of Green tea is performed using HPLC, GPC or TLC. The primary bioactive components fall into the following chemical categories: alkaloids, benzonoids, terpenes, sesquiterpene, diterpenes, monoterpenes and carbohydrates. Most of the studies have focused on the catechin (Goto et al., 1996, *J. Chrom.* A749:295-299). The TLC analysis of Green tea is performed as described in a recent publication (Minpeigen, 1991, *Phytotherapy Res.* 5:239-240).

26. EXAMPLE: HAWTHORN, *Crataegus laevigata*

26.1 PLANT SOURCE/BACKGROUND

The botannical Hawthorn is based on the dried leaves of flowers of *Cratigus laevigata* which is harvested both wild or from cultivated populations in Europe. It is also used in China and in America. Hawthorns are members of the rose family.

In traditional Chinese medicine, the Hawthorn's fruits are used for digestion, to promote stomach function, to stimulate blood circulation and various stomach disorders. The traditional Chinese uses also include hypertension, hyperlipdemia and coronary heart disease.

26.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Hawthorn. Some of the major products are Heartcare™ and Wild European Hawthorn™, by Murdock Madaus Schwabe (Springville, Utah), and

Hawthorn Ticture, by Natural Factors Nutritional Products, Ltd. (Burnaby, British Columbia, Canada). Hawthorn leaves and flowers powdered extract is available from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany), and is standardized to contain 1.8-2.8% vitexin-hyperoside.

26.3 CLINICAL INDICATIONS

In Europe, the fruits, flowers and leaves are combinations used as astringents, antispasmodics, cardiotonics, diarrhetics, hypotensives and antiarrhythmics. American Indians also have used Hawthorn for gastrointestinal and circulation disorders.

Hawthorn has been approved for use in Germany for cardiac insufficiency and bradyarrhythmia (German Commission E Monograph B Anz 1, dated January 3, 1989). Clinical indications of Hawthorn also include increasing cardiac performance, treating senile heart conditions, treating mild forms of angina pectoris and dysrhythmia and lowering blood pressure (Occhiuto et al., 1986, *Planta Med.* 20:52; Stepka and Winters, 1973; *Lloydia*, 36:436). Clinical studies have also reported that it has an anti-arrhythmic effect, increases myocardium tolerance to oxygen deprivation and stimulus revascularization after myocardial ischemia (Guendjv, 1977, *R. Arnzeim. Forch*, 27:1576).

26.4 FRACTIONAL ANALYSIS

Fractional analysis of Hawthorn is performed first by a separation of the aqueous and organic layers (Thompson et al., 1974, *J. Pharm. Sci.* 63:193-6), the fractions are then separated further on a C-18 column.

26.5 BIOLOGICAL ANALYSIS

The biological analysis of Hawthorn is performed using a variety of methods including a thromboxin A2 assay, measurement of coronary vasodilation, measurement of cardiac muscle contractility and measurement of ventricle output velocity. The thromboxin A2 assay is performed as follows.

26.6 THROMBOXANE A_2

In this assay the ability of Hawthorn extracts and fractions to modulate the binding of [^{3}H]SQ-29548 to thromboxane A_2 receptors is measured . Platelets of male or female New Zealand derived albino rabbits weighing 2.5-3 kg are prepared in modified Tris-HCl buffer (pH 7.2) using standard techniques. A 2 mg aliquot of membrane is incubated with 3 nM [^{3}H] SQ-29548 for 45 minutes at 25°C. Non-specific binding is estimated in the presence of 1 μM BM 13505. Membranes are filtered and washed 3 times and the filters are counted to determine [^{3}H] SQ-29548 specifically bound. Compounds are screened at 10 μM (Saussy et al., 1986, *J. Biol. Chem.* 261: 3025-3029; Hedberg et al., 1988, *J. Pharmacol. Exp. Ther.* 245: 786-792).

Assay Reference Data:

K^d	4.2 nM
B^{max}:	960 fmol/mg protein
Specific Binding:	95%

Compound	IC_{50} (nM)	Ki (nM)	nH
*BM-13505	18	11	0.9
SQ-29548	6.6	3.9	1.1
U-46619	34	20	1.1

*Indicates standard reference agent used.

BM-13505= deltroban; SQ29548=(Is-[1α,2β{5Z},3β,4α])-7-(3-[{2-phenylamino) carbonyl}hydrazino]methyl)-7-oxabicyclo[2.2.1.]hepy-2-yl-5-heptanoic acid; U-46619=11α,9α-epoxymethano-PGH_2

The myocardial effects of Hawthorn extracts may be analyzed using published procedures (Schusselr et al., 1995, *Annzeim. Forch. Drug Res.* 45:11). In one study isolated guinea pig hearts are analyzed for coronary flow, heart rate and left ventricle blood pressure as well as velocity of contraction and relaxation. The results of the study of the extracts are compared with hyperoside luteolin-7-glucoside and rutin.

Hawthorn may also be studied for its protective effect against myocardial ischemia using procedures described in the literature (Lianda et al., 1984, *J. Trad. Chinese Med.* 4:289-292). Hawthorn is studied using cultured rat heart cells and rat heart cells deprived of oxygen and glucose.

Previous workers have shown that vitexin rhamnoside is active as a heart protective agent on the cultured cell assay.

26.7. CHEMICAL ANALYSIS HPLC

C. laevigata and *C. monogyna* contain flavonoids, including hyperoside (hyperin), quercetin, vitexin, vitexin-4'-L-rhamno-D-glucoside, vitexin-4'-L-rhamnoside, vitexin-4'-7-di-D-glucoside, rutin, quercetin-3-rhamno-galactoside, and others. Xanthine, amines, proanthocyanidins are also present. (Leung and Foster, 1996).

The chemical analysis of the hawthorn is performed using reverse phase HPLC as described in the St. John's Wort experiments above.

27. EXAMPLE: IVY LEAF, *Hederae folium*

27.1 PLANT SOURCE/BACKGROUND

The ivy is native throughout Europe and the Mediterranean. It is typically marketed in the form of the extract. It has been traditionally regarded as an anti-septic, astringent, cathartic, contraceptive, emetic, laxative, purgative, stimulant, vasoconstrictor, vasodilator and vermifuge. Ivy is also used for rheumatism sclerosis, scrofula and toothache.

27.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

Commercially available ivy sources include Prospan™ (Engelhard, Germany), Hedelix™ (Krewel Meuselbach, Germany) and Bronchofortan™.

27.3 CLINICAL INDICATIONS

Ivy has been approved in the German monographs for use in inflammation of the respiratory passages, treatment of chronic inflammatory bronchial conditions (German Commission E Monograph B Anz No. 122, dated July 6,1988). The typical dose of ivy leaf is 0.3 grams of the drug or corresponding extract. It also has activity for osteoarthritis and GI distress. Ivy has expectorant activities diaphoretic and astringent activities. It may be used for dyspeptic complaints.

27.4 FRACTIONAL ANALYSIS

Fractional analysis of the ivy leaf extract is performed using reverse phase chromatography as described in the St. John's Wort experiments above.

27.5 BIOLOGICAL ANALYSIS

The biological analysis is performed using the ileum model to study secretolytic action and spasmolytic effects.

27.6 CHEMICAL ANALYSIS HPLC

The components present in Ivy include saponins (2.5-6%), α-hederin, oleanolic-acid glycosides, hederacoside C, rhamnose; flavonol glycosides, kaempferol 3-rutinoside; traces; sterols stigmasterol, sitosterol, cholesterol, campesterol, α-spinasterol, and 5α-stigma-7-en-3β-ol; scopolin, chlorogenic acid, caffeic acid, the sesquiterpene hydrocarbons germacrene, β-elemene, lobinol and antigenic catechols (Bisset, 1994).

28. EXAMPLE: KAVA, *Piper methysticum*

28.1 PLANT SOURCE/BACKGROUND

Kava is also known as kava kava. It is a deciduous shrub which occurs throughout the South Pacific from Hawaii to New Guinea. The part that is used for the drug is the root stock.

28.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Kava. Some of the major products are Polynesian Kava Root, by Murdock Madaus Schwabe (Springville, Utah), Antares™ (Krewel Meuselbach, Germany), Laitan™ (Schwabe, Germany) and Kavatrol™, by Natrol (Chatsworth, California). Kava root powdered extract is also available from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany), and is standardized to contain 30% kavapyrones.

28.3 CLINICAL INDICATIONS

Kava has many biological activities. It has been used as a sedative for insomnia, for nervousness, for stress and as a muscle relaxant. It has anti-spasmodic and anti-convulsive activities. The primary indications for kava are as a non-opiate analgesic.

Kava has been approved for use in conditions of nervous anxiety, stress and unrest. Kava is combined with pumpkin seed oil, *Cucurbita semen*, for use in the treatment of irritable bladder syndrome. Kava has been approved in the German Commission E Monograph No. 101 (June 1, 1990).

28.4 FRACTIONAL ANALYSIS

The fractional analysis on kava is performed using reverse-phase chromatography on a C-18 column or flash chromatography on silica gel performed as described above, in the St. John's Wort and the Saw Palmetto sections above.

28.5 BIOLOGICAL ACTIVITY ANALYSIS GABA

The biological analysis for Kava's activity is performed by studying the binding to the GABA receptors both peripheral and central, the serotonin re-uptake assay and binding to dopamine receptors.

The analysis for binding to the GABA-A and GABA-B receptors and benzodiazepin receptors is performed using published procedures (Davies et al., 1992, *Pharm. and Toxin.* 71:120-126). In addition, the binding of the Kava extracts is also studied using a published displacement assay for the GABA-A receptor (Jussoifie et al., 1994, *Psychopharmacology* 116:469-474).

28.6 CHEMICAL ANALYSIS

Kava contains from 3-20% kavalactones. The kavalactones in the resin are α-pyrones bearing a methoxy group at carbon 4 and an aromatic styryl moiety at carbon 6, including kavain, 7,8-dihydrokavain, 5,6-dehydrokavain, yangonin, 5,6,7,8-tetrahydroyangonin, methysticin, dihydromethysticin, 5,6-dehydromethystincin, 5,6-dihydroyangonin, 7,8-dihydroyangonin, 10-methoxy-yangonin, 11-methoxy-yangonin, 11-hydroxy-yangonin, hydroxykavain, and 11-methoxy-12-hydroxy-dehydrokavain. The rootstock also contains flavokavins A and B; the alkaloid pipermethystin; cepharadione A; ketones including cinnamalaketone, and methylene dioxy-3,4-cinnamalaketone; and an alcohol, dihydrokavain-5-ol. (Leung & Foster, 1996).

29. EXAMPLE: LICORICE, *Glycyrrhiza glabra*

29.1 PLANT SOURCE/BACKGROUND:

Licorice is a four to five foot shrub which grows in sub-tropical climates. Most commercial licorice is of the variety *G. glabara*. The drug consists of the dried rhizome and roots. It is a member of the pea family.

29.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of licorice. Powdered licorice root extract is available from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany). Licorice dry extract, standardized to contain 5% glycyrrhizinic acid, is available from Indena s.a. (Milan, Italy), Lakriment™ (Knoll, Germany), Suczulen™ (Dolorgiet, Germany) and Licorice Root 500™ is available from Natural Factors Nutritional Products, Ltd. (Burnaby, British Columbia, Canada).

29.3 CLINICAL INDICATIONS

The clinical indications for licorice are used as an anti-ulcer medication, anti-inflammatory, anti-arthritic, as an expectorant and as anti-infective agent. The typical licorice dose is 5-15 g of finely cut or powdered root (calculated to contain 200-600 mg of glycyrrhizin). The most common use is in fusions for the treatment of gastric-duodenal ulcers. Alternatively, the root juice (0.5-1 g for respiratory tract inflammation or 1.5 to 3 g for ulcers) are used. It has been approved by the German Commission E Monograph No. 90 (May 15, 1985, updated March 13, 1990 and April 4, 1991).

Licorice constituents have been shown to have anti-HIV activity (Hatano et al., 1988, *Chem. Pharm. Bull.* 36(6):2286-2288). Licorice has also been shown to be active against *Streptococcus* which is responsible for dental caries (Segal et al., 1985, *J. Pharm. Sci.* 74:79-81).

29.4 FRACTIONAL ANALYSIS

The fractional analysis of the components of licorice is performed using a reverse-phase C-18 column as described above.

29.5 BIOLOGICAL ACTIVITY ANALYSIS

The biological analysis of the total licorice extract and the individual components of licorice is performed using the cyclooxygenase assay and the lipoxygenase assay and anti-prostaglandin assays described above. Another bioassay that is performed is the 11-β hydroxy steroid dehydrogenase assay (MacKenzie et al., 1990, *J. Clin. Endocrinol. Metab.* 70:1637-1643).

29.6 CHEMICAL ANALYSIS HPLC

It contains as its major bioactive principle the triterpene glycoside glycyrrhizin (also known as glycyrrhizic or glycyrrhizinic acid) in concentrations ranging from 1 to 24%, depending on sources and methods of assay. Some have reported a 10-fold difference in glycyrrhizin values due solely to different assay methods has been reported. Glycyrrhizin on hydrolysis yields glycyrrhetinic (or glycyrrhetic) acid and two molecules of glucuronic acid.

Other constituents of licorice include flavonoids and isoflavonoids (licoflavonol, kumatakenin, licoricone, glabrol, glabrone, glyzarin, licoisoflavones A and B, licoisoflavanone, glycyrol, formononetin, liquiritigenin, liquiritin, neoliquiritin, rhamnoliquiritin, glyzaglabrin, 4'-7'-dihydroxyflavone, glaranine, etc.), chalcones (isoliquiritigenin, isoliquiritin, neoisoliquiritin, licuraside, rhamnoisoliquiritin, echinatin, licochalcones A and B, 4-hydroxychalcone, etc.), coumarins (umbelliferone, herniarin, liqcoumarin, glycerin, etc.), triterpenoids (liquiritic acid, glycyrrhetol, glabrolide, isoglabrolide, licoric acid, β-amyrin, 18-β-glycyrrhetinic acid, etc.), sterols (β-sitosterol, stigmasterol, 22,23-dihydrostigmasterol etc.), 2-20% starch, 3-14% sugars (glucose and sucrose), lignin, amino acids (proline, serine, aspartic acid, etc.), amines (asparagine, betaine, choline), gums, wax, a volatile oil consisting of many aroma chemicals (including acetol, 2-acetylfuran, propionic acid, 2-acetylpyrrole, furfuryl alcohol, benzaldehyde, pentanol, hexanol, *trans*-hex-3-en-1-ol, oct-1-en-3-ol, linalool, linalyl oxide, α-terpineol, butyrolactone, thujone, and fenchone, among others, none of which alone can account for the licorice flavor, and others. (Leung & Foster, 1996).

The chemical analysis of licorice fractions is performed by HPLC.

30. EXAMPLE: MILK THISTLE, *Silybum marianum* also *Carduus marianus, Carduus marianum*

30.1 PLANT SOURCE/BACKGROUND

Milk Thistle is a plant indigenous to Kashmir but is found in North America from Canada to Mexico. Milk Thistle is sold as a botanical in the form of the whole seeds and as an extract. It is traditionally used as a liver protectant.

30.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

Milk Thistle is another extremely popular botanical product. Milk Thistle 250™ is available from Natural Factors Nutritional Products, Ltd. (Burnaby, British Columbia, Canada) and milk thistle powdered extract, standardized to contain 70%-80% Silymarin is available from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany). Milk Thistle extracts are also available from Bionorica (Germany), Madaus AG (Germany), and Klinger (Germany). The following companies also supply Milk Thistle: Herbal Choice-Botalia, NaturaLife, Herb Pharm, Nature's Herbs, PhytoPharmica, Legalon™ (Madaus, Germany), Silimarit™ (Bionorica, Germany), Permixon™ (Pierre Fabre, Germany) and Nature's Way.

30.3 CLINICAL INDICATIONS

(1) Hepatoprotective, free radical scavenger, antioxidant, cirrhosis, hepatitis, dyspeptic complaints, liver/gall bladder complaints, loss of appetite

30.4 FRACTIONAL ANALYSIS

The fractional analysis on Milk Thistle is performed using reverse-phase chromatography on a C-18 column or flash chromatography on silica gel. Both methods are performed as described above in the St. John's Wort and the Saw Palmetto sections, respectively.

30.5 BIOLOGICAL ACTIVITY ANALYSIS

The biological analysis is performed studying cultured hepatocytes, c-AMP, β-Glucuronidase and liver enzyme levels.

30.5.1 ASSAY FOR INHIBITION OF β-GLUCURONIDASE

β-glucuronidase is a typical lysosomal enzyme which increases in the blood after liver damage or liver cancer. Thus, hepatoprotective effects of milk thistle and its components is quantified by their inhibitory effects on this enzyme. The enzyme can be prepared from human feces as described (Kim et al. 1994 (*Biol. Pharm. Bull.* 17: 443-445). For measuring the enzyme activity, the enzyme is incubated at 37°C (50 minutes) with 20 ul of 10 mM of p. nitophenyl-β-glucuronide in 0.1 M phosphate buffer (pH 7.0) in presence or absence of inhibitor. The reaction is stopped by the addition of 0.25 N NaOH and measuring the absorbance of released p. nitrophenol at 405 nm.

30.5.2 ASSAY FOR THE INHIBITION OF CYCLIC-amp PHOSPHODISTRASE (PDE)

In this assay C-AMP (0.1 mM) is incubated with PDE in a 40 mM Tris-Hel buffer (pH 7-8) for 10 minutes at 25°C in the presence of various amounts of milk thistle extracts and fractions as described by Koch et al., 1985 (*Meth. Find Exp. Clin. Pharmacol.* 1: 409-413). The reaction is stopped by the addition of chilled perchloric acid. The protein precipitate is centrifuged and the supernatant is assayed by HPLC (reversed phase chromatography). The inhibition of the enzyme is studied by reduced conversion of C-AMP to AMP.

30.6 CHEMICAL ANALYSIS TLC, HPLC

A flavanolignan complex, silymarin, was first isolated from the seeds in 1968. Silymarin (4-6% in ripe fruits) consists primarily of three flavanolignans, silybin

(silibinin), silychristin (silichristin), and silidianin. Other flavanolignans include dehydrosilybin, 3-desoxysilichristin, deoxysilydianin (silymonin), siliandrin, silybinome, silyhermin, and neosilyhermin. Other constituents include apigenin, silybonol; a fixed oil (16-18%), consisting largely of linoleic and oleic acids, plus myristic, palmitic, and stearic acids; betaine hydrochloride, triamine, histamine, and others (Leung and Foster, 1996). Milk Thistle is analyzed using TLC and HPLC as described above.

31. EXAMPLE: PASSION FLOWER, *Passiflora incarnata*

31.1 PLANT SOURCE/BACKGROUND

The Passion Flower is native to the Americas, and is cultivated in both subtropical and tropical regions. The drug is produced in the U.S., India and the West Indies.

31.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Passion Flower extracts. Passion flower dry extract is available from Indena s.a. (Milan, Italy), and is standardized to contain 3.5% flavonoids calculated as isovitexine. Powdered extracts are also available from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany) and is standardized to contain 4% flavonoids. It is also available from Passifloradragees Alsitan™ (Alistan, Germany).

31.3 CLINICAL INDICATIONS

The Passion Flower is used as a sedative treating anxiety, for difficulties in sleeping, restlessness, various nervous disorders. It is also used as an spasmolytic agent (Paris, 1963, *Ann. Pharm. Franc.* 21:389). The typical dosing regime is 4-8 g of drug taken daily. The neuropharmacology

studies have indicated a central nervous system effect (Speroni and Minghetti, 1988, *Planta Med.* 54:488).

31.4 FRACTIONAL ANALYSIS

The fractional analysis of Passion Flower is performed using reverse-phase chromatography on a C-18 column or flash chromatography on silica gel. Both are performed as described above in the St. John's Wort and the Saw Palmetto sections, respectively.

31.5 BIOLOGICAL ACTIVITY ANALYSIS

The biological analysis is for MAO, GABA A (peripheral, central) and the experiments are performed as described above.

31.6 CHEMICAL ANALYSIS HPLC

Passion Flower contains small and highly variable amounts (<0.01-0.09%) of indole alkaloids, consisting mainly of harman, with lesser amounts of harmol, harmaline, harmine, and harmalol. Presence of the last four alkaloids have been disputed. Other constituents present include flavonoids (isovitexin 2"-β-D-glucoside, isoorientin 2"-β-D-glucoside, apigenin, luteolin, quercetin, kaempferol, schaftoside, isoschaftoside, saponaretin, saponarin, vitexin, orientin, and rutin; a cyanogenic glucoside, gynocardin (0.01%); sugars (raffinose and sucrose predominant); sterols (stigmasterol and sitosterol); n-non-acosane, and gum, among others. Maltol and ethyl maltol have been isolated from the plant. The coumarins umbelliferone and scopoletin have been detected in the root (Leung and Foster, 1996). It is analyzed by HPLC.

32. EXAMPLE: PUMPKIN, *Cucurbitae semen*

32.1 PLANT SOURCE/BACKGROUND

The Pumpkin is variously known as vegetable marrow, summer and autumn squash, and is native to North America, but is cultivated worldwide. The dosing format requires use of whole or powdered seeds.

32.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Pumpkin seed extracts. Botanicals International, a division of Zuellig Botanicals, Inc. (Germany) offers two varieties: powdered extract with .02% phytosterols, and a soft extract with .5% phytosterols and 70% fatty acids. Also, pumpkin seed lipophilic extract is available from Indena s.a. (Milan, Italy). It is also available as Prosta Fink™ (Fink, Germany) and Cysto-Uregenin™ (Madaus, Germany).

32.3 CLINICAL INDICATIONS:

The main indication is for treating problems of micturition, especially associated with benign prostrate hyperplasia (BPH). The clinical use is largely empirical, but there are reliable reports (Lutzelburger, *Artzl. Praxis*, 1974, 76:3278; Haefele, *Artzl. Praxis*, 1977, 79:3321). The average daily dose is 10 g of seeds.

32.4. FRACTIONAL ANALYSIS

The fractional analysis on Pumkin seeds and extracts is performed using reverse-phase chromatography on a C-18 column or flash chromatography on silica gel. Both are performed as described above in the St. John's Wort and the Saw Palmetto sections, respectively.

32.5 BIOLOGICAL ACTIVITY ANALYSIS

For the clinical indication of BPH, the primary assays for the anti-inflammatory activity are cyclooxygenase-1 and -2 and 5-lipoxygenase. The experiments are performed as described in the Saw Palmetto section above. The other relevant bioassays for the BPH clinical condition are the androgen receptor binding assay and the 5-α-reductase assay. These assays are performed as described above in the Saw Palmetto section above.

32.6 CHEMICAL ANALYSIS

Pumpkin seeds contain 1% steroids, especially 24β-ethyl-5α-cholesta-7,25(27)-dien-3β-ol and 24β-ethyl-5α-cholesta-7-trans-22,25(27)-trien-3β-ol as essential components, and to some extent also sterol glucosides and Δ^5- and Δ^8-sterols; tocopherols (vitamin E); trace elements, particularly selenium, manganese, zinc, and copper; 35-40% fixed oil; ca/ 30% pectins; ca. 25-30% protein (Bisset, 1994).

33. EXAMPLE: PYGEUM, *Pygeum africanum*

33.1 PLANT SOURCE/BACKGROUND

The plant is found mainly in Africa, with production of the bark in Cameroon. Extraction of ground dried bark with organic solvents and drying provides a concentrated powdered drug of varying constitution (Bonati, *J. Ethnopharmacol.*, 1991, 32:195-7).

33.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Pygeum extracts. One major supplier is Indena s.a. (Milan, Italy) which offers pygeum purified soft extract, which is standardized to contain 13% total sterols calculated as beta-sitosterol. Tadenan™ by DEBAT Laboratories (Garches, France), Pronitol™by Inofarma (Madrid, Spain), and Pygeum

Capsules™ by Murdock Madaus Schwabe (Springville, Utah) are also popular.

33.3 CLINICAL INDICATIONS

The main indication is similar to Pumpkin and Saw Palmetto, for treating problems of micturition, especially associated with benign prostrate hyperplasia (BPH). A recently reported clinical trail of 56 days treatment in 134 patients suffering from BPH, and treated with 25 mg of the drug in combination with stinging nettle was deemed both safe and efficacious. (Krzeski, Kazon, Borkowski, Witeska and Kuczera, 1993, *Clin. Ther.*, 15:1011-20).

33.4 FRACTIONAL ANALYSIS

The fractional analysis on Pygeum is performed using reverse-phase chromatography on a C-18 column or flash chromatography on silica gel and are performed as described above in the St. John's Wort and the Saw Palmetto sections, respectively.

33.5 BIOLOGICAL ACTIVITY ANALYSIS

For the clinical indication of BPH, the primary assays for the anti-inflammatory activity are cyclooxygenase-1 and -2 and 5-lipoxygenase bioassays. The experiments are performed as described in the Saw Palmetto section above. The other relevant bioassays for the BPH clinical condition are the androgen receptor binding assay and the 5-α-reductase assay. These assays are also performed as described above in the Saw Palmetto section. Muscle contractility is also measured.

33.6 CHEMICAL ANALYSIS HPLC, GC

The chemical analysis is performed by HPLC and GC. The components include alkanes, fatty acids, sterols, and triterpenes.

34. EXAMPLE: ROSEMARY, *Rosemarinus officinalis*

34.1 PLANT SOURCE/BACKGROUND

The Rosemary plant is native in the Mediterranean region, and is cultivated in many countries. The drug is produced in Spain, Morocco, Yugoslavia and Tunisia. Rosemary oil is produced by steam distillation of the leaves and leafy stems. The leaves are used fresh or dried, and can be extracted using ethanol.

34.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

Rosemary leaf powder extract is available from Botanicals International, a division of Zuellig Botanicals, Inc. (Germany).

34.3 CLINICAL INDICATIONS

The main indication is for use as a carminative and stomachic in digestive upsets, flatulence, distension, appetite and gastric secretion stimulation. It is also used as a choleretic. Externally, it is used in the form of an oil or linament for treatment of rheumatism as well as in local stimulation of circulation. The average daily does is 2-6 g of drug or 10-20 drops of the essential oil.

34.4 FRACTIONAL ANALYSIS

The fractional analysis of the components of Rosemary is performed using a reverse-phase C-18 column as described above.

34.5 BIOLOGICAL ACTIVITY ANALYSIS

The biological anlysis is performed using the following assays, Aldol-reductase, rat and mouse diabetes, db db mice, Ob /Ob mice, Zucker rats. The Aldol Reductase assay is performed as follows.

34.5.1 ALDOSE REDUCTASE

Aldose reductase, a member of the monomer NADPH-dependent aldo-keto reductase, is a rate-limiting enzyme in the polyol pathway which catalyses the reduction of various aldehydes. This includes reduction of the aldehyde form of glucose to its corresponding sugar alcohol sorbitol. Accumulation of sorbitol has been reported in the lens, nerve, kidney and retina of diabetic animals. Large amounts of sorbitol cause osmotic disruption which may be one of the etiologic factors in the pathogenesis of some diabetic complications.

Aldose reductase from rat lens is partially purified by tissue homogenization and centrifugation. The compound or vehicle, enzyme, NADPH and assay buffer are preincubated at 25°C for 3 minutes and absorbance is observed at 340 nm for the initial zero time value. The reaction is then initiated by addition of DL-glyceraldehyde and incubation is continued for 8 minutes at 25°C at which time the final absorbance is noted. Enzyme activity is determined by the difference between initial and final absorbance. Compounds are screened at 100 μM (DeRuiter et al., 1993, *J. Enzyme Inhibition* 7: 249-256; Kubo et al., 1994, *Biol. Pharm. Bull.* 17: 458-459). Standard reference agents are carbidopa and benserazide.

34.6 CHEMICAL ANALYSIS HPLC, GC

Rosemary contains about 0.5% volatile oil; flavonoids (diosmetin, diosmin, genkwanin, genkwanin-4'-methyl ether, 6-methoxy-genkwanin, luteolin, 6-methoxyluteolin, 6-methoxyluteolin-7-methyl ether, hispidulin, apigenin, etc.);

phenolic acids (rosmarinic, labiatic, chlorogenic, neochlorogenic, and caffeic acids); carnosic acid; rosmaricine and isorosmaricine (reaction products of carnosic acid); triterpenic acids (mainly ursolic and oleanolic acids, with traces of 19α-hydroxyursolic, 2β-hydroxyoleanolic, and 3β-hydroxyurea-12,20(3)-dien-17-oic acids); rosmanol, 7-ethoxyrosmanol, betulinic acid, and carnosol; and others. The essential oil contains mainly monoterpne hydrocarbons (α- and β-pinenes, camphene, limonene, etc.), cineole (eucalyptol), and borneol, with camphor, linalool, verbenol, terpineol, 3-octanone, and isobornyl acetate also present. (Leung and Foster, 1996).

The volatile oil includes monoterpene hydrocarbons, camphor, borneol and cineole and flavonoids.

35. EXAMPLE: SIBERIAN GINSENG, *Eleutherococcus senticosus*

35.1 PLANT SOURCE/BACKGROUND

Siberian ginsing is a relative of Asian ginsing that was introduced by Russian scientists. The herbal medicine is based on the root.

35.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

Siberian Ginseng in various forms is available as follows: Natural Life, Herbal Harvest, Herbal Choice-Botalia, Herb Pharm, Eleu-Kokk™ (Pharmaton, Germany) and Vital-Kapseln-ratiopharm™ (Ratipharm, Germany).

35.3 CLINICAL INDICATIONS

Siberian Ginsing has many clinical indicatins including anticancer properties immunostimulatory properties and lowering blood pressure. It is also reported as a general tonic.

35.4 FRACTIONAL ANALYSIS

The fractions are prepared as described in the Mistletoe section above. Alternately, published procedures are used (Bauer and Remiger, 1989, *Planta Med.* 55:367-371; Steinmuller et al., 1993, *J. Immunopharmacol.* 15(5):605-614).

35.5 BIOLOGICAL ACTIVITY ANALYSIS

The biological analysis for the anticancer clinical indication is performed as described in the Mistletoe section above.

35.6 CHEMICAL ANALYSIS

The chemical analysis is done by HPLC. The components include eleutherosides, ligands and sterols.

36. EXAMPLE: STINGING NETTLE, *Urtica dioica*

36.1 PLANT SOURCE/BACKGROUND

This plant has almost worldwide distribution, and is produced in southeastern Europe. The drug is taken externally as a cold expressed oil as well as internally as a tea made from finely divided plant material.

36.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

Fresh freeze dried nettles are available from Ecclectic Institute (Sandy, Oregon) as well as from Bionorica (Germany). The root (urologic agent), is available from Bazoton™ (Kanoldt, Germany), Prostaforton™ (Sanofi Winthrop, Germany) and leaves (anti-rheumatice) are available from Rheuma-Heck™ (Krewel Meuselbach, Germany).

36.3 CLINICAL INDICATIONS

The main use is as a diuretic, although there are other uses as a promoter of wound healing, and in treating biliary complaints (Kirchhoff, *Z. Phytotherap*, 1983, 4:621). The average daily dose is 3-4 teaspoons of nettle herb in 150 ml of hot water, taken 3-4 times daily.

36.4 FRACTIONAL ANALYSIS

The fractional analysis of the components of Stinging Nettle is performed using a reverse-phase C-18 column as described above.

36.5 BIOLOGICAL ACTIVITY ANALYSIS

The biological analysis is done as described for Saw Palmetto above.

36.6 CHEMICAL ANALYSIS HPLC

β-sitosterol and tannins, recently scopoletin, β-sitosteryl 3-β-D-glucoside, and other sterols and steryl glucosides have been isolated from the drug. Phenylpropanes, including homovanillyl alcohol and the corresponding glucoside, as well as lignans of the relatively rare monoepoxylignan type, among them neo-olivil and derivatives, have been detected in the drug. An array of polyphenols and their methyl ethers is present. Several monoterpene diols in free and glucosidic form have been isolated. Lectins (0.1-0.2%), the so called UDA (Urtica dioica agglutinin), and polysaccharides (both acid and neutral), as well as 9-hydroxyocatadeca-10-*trans*, 12-cis-dienoic acid, have also been found (Bisset, 1994).

37. EXAMPLE: Valerian, *Valeriana officianalis*

37.1 PLANT SOURCE/BACKGROUND

The Valerian plant is native to Europe and Asia and naturalized in North America. The drug is cultivated in the U.S., Japan, and Europe. Most common usage is as a root, rhizomes and stolons and taken as a tea. An essential oil and tincture is also produced by ethanolic extraction of the roots.

37.2 COMMERCIAL SUPPLIERS/PRODUCT NAMES

There are a variety of commercial suppliers of Valerian extracts. One major supplier is Indena s.a. (Milan, Italy) which offers valerian dry extract which is standardized to contain 0.8% valerenic acids. Other products include Calmaid™ and Valerian Nighttime™ by Murdock Madaus Schwabe (Springville, Utah). Flora Laboratories, Trout Lake Farm, PhytoPharmica, Herbal Choice-Botalia, Botalia Gold, Herb Pharm, Nature's Way and Shaklee.

37.3 CLINICAL INDICATIONS

The clinical indications associated with Valerian are as follows: calmative, sleep-promoting agent, calmative, hepatoprotection, cirrhosis, hepatitis, immunomodulatory activity, hyperlipidemia, sedative, stress, nervousness and insomnia.

37.4 FRACTIONAL ANALYSIS

The fractional analysis of the components of Valerian is performed using a reverse-phase C-18 column as described above. The fractions are also prepared as described in the Mistletoe section above. Alternately, published procedures are used (Bauer and Remiger, 1981; Steinmuller et al., 1993)

37.5 BIOLOGICAL ACTIVITY ANALYSIS

Valerian is analyzed for CNS receptor, GABA-A (central, peripheral), and $5HT_1$ receptor activity as described above. Depression behavior is studied using the animal model above.

37.5.1 BIOASSAY FOR AMINOBUTRIC ACID-A (GABA-A) RECEPTOR BINDING

Male rats are sacrificed by decapitation, their cortices are removed and homogenized in Potter-Eleveheim homogenizer in 10 volumes of cold 0-32 M sucrose (pH 7.4). The homogenized material is centrifuged at 100 g for 10 minutes. Then the supernatant is dentrifuged at 20,000 g for 20 minutes and the pallet is resuspended in 20 volumes of fresh distilled water cooled nd recentrifuged at 8,000 x g for 20 minutes. The supernatant and 'buffy-coat' are centrifuged at 50,000 x g for 20 minutes to obtain GABA-A receptor as pallet. The binding assays are performed in 50mm Tris-HCL pH 7.4 buffer. The effects of Valerian extract and its fractions on binding of ^{3}H-muscimol to GABA-A receptor after 30 minutes incubation at 0°C are quantified according to the method of Bernasconi and Morazzoni, 1993 (Fitoterapia, vol. LXIV, 291-299).

37.6 CHEMICAL AYNALYSIS HPLC, TLC, supercritical carbon dioxide

Common Valerian contains as its primary bioactive constituents several iridoid compounds called valepotriates including valtrates (valtrate, valtrate isovaleroxhydrin, acevaltrate, valechlorine, etc.), didrovaltrates (didrovaltrate, homodidrovaltrate deoxydodidrovaltrate, homodeoxydodidrovaltrate, isovaleroxyhydroxydidrovaltrate, etc.), and isovaltrates (isovaltrate, 7-epideacetylisovaltrate, etc.) valtrate and didrovaltrate are the major valepotriates. In addition, it contains valerosidatum (an iridoid ester glycoside) and a volatile oil (0.5-2%) consisting of many components including bornyl

acetate and isovalerate (major compounds), caryophyllene, α- and β-pinenes, valerenal, valerenic acid, valeranone, β-ionone, eugenyl isovalerate, isoeugenyl isovalerate, patchouli alcohol, valerianol, borneol, camphene, β-bisabolene, ledol, isovaleric acid, and terpinolene, among others. Common valerian also contains several alkaloids including actinidine valerianine, valerine, and chatinine. Other constituents present in common valerian include choline (ca. 3%), methyl 2-pyrrolyl ketone, chlorogenic acid, and caffeic acid; β-sitosterol; tannins; gums; and others. Indian valerian is generally reported to contain similar constituents as common valerian including valepotriates, valerosidatum, and a volatile oil. In addition, it contain 2A'''-O- and 3'''-O-2-methylbutyryl esters of acetylated linarin (Leung and Foster, 1996).

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed since these embodiments are intended as illustration of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Throughout this application various publications and patents are cited in parenthesis. Their contents are hereby incorporated by reference into the present application.

We claim:

1. A method for making a pharmaceutical grade botanical drug, the method comprising the steps of:

providing a botanical material which has a given biological activity, said botanical material comprising a plurality of components;

separating a representative aliquot of the botanical material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active component;

determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the representative aliquot; and

comparing the bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade botanical drug to determine whether the botanical material is a pharmaceutical grade botanical drug.

2. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein one or more of the marker fractions contain one active component.

3. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the method comprises the additional steps of:

determining the amount of the active components in at least one of the marker fractions to provide a quantitative compositional fingerprint of the representative aliquot and

comparing the quantitative compositional fingerprint of the representative aliquot to a quantitative compositional fingerprint standard which has been established for a given pharmaceutical grade botanical drug to determine whether the botanical material is a pharmaceutical grade botanical drug.

4. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the method comprises the additional steps of:

determining a total bioactivity of the representative aliquot of the botanical material and

comparing the total bioactivity of the representative aliquot with that of a total bioactivity of the standard to determine whether the botanical material is a pharmaceutical grade botanical drug.

5. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the botanical material is an extract made from plant material.

6. The method for making a pharmaceutical grade botanical drug according to claim 5, wherein the botanical material is a supercritical carbon dioxide extract.

7. The method for making a pharmaceutical grade botanical drug according to claim 5, wherein the botanical material is an ethanolic extract.

8. The method for making a pharmaceutical grade botanical drug according to claim 5, wherein the botanical material is an aqueous or organic extract.

9. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the botanical material is a seed oil.

10. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the botanical material is a powdered plant material.

11. The method for making a pharmaceutical grade botanical drug according to claims 1, 2, 3 or 4, wherein the botanical material is selected from the group consisting of Aloe, Bilberry, Black Cohosh, Chamomile, Chaste tree, Chestnut, Echinacea, Evening Primrose, Feverfew, Garlic, Ginger, Ginkgo, Ginseng (Asian), Goldenseal, Green tea, Guggulipid, Hawthorn, Ivy, Kava, Licorice, Milk Thistle, Passion Flower, Pumpkin, Pygeum, Rosemary, Siberian Ginseng, Saw Palmetto, St. John's Wort, Stinging Nettle and Valerian.

12. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Aloe.

13. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Bilberry.

14. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Black Cohosh.

15. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Chamomile.

16. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Chestnut.

17. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Echinacea.

18. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Evening Primrose Oil.

19. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Feverfew.

20. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Garlic.

21. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Ginger.

22. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Ginkgo.

23. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Ginseng (Asian).

24. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Goldenseal.

25. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Green tea.

26. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Hawthorn.

27. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Ivy leaf.

28. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Kava.

29. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Licorice.

30. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Milk Thistle.

31. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Passion Flower.

32. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Pumpkin.

33. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Pygeum.

34. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Rosemary.

35. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Saw Palmetto.

36. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Siberian Ginseng.

37. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is St. John's Wort.

38. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Stinging Nettle.

39. The method for making a pharmaceutical grade botanical drug according to claim 11, wherein the botanical material is Valerian.

40. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the botanical material is a mixture of plant materials.

41. The method for making a pharmaceutical grade botanical drug according to claim 40, wherein the mixture of plant materials is a mixture of Saw palmetto and Pumpkin.

42. The method for making a pharmaceutical grade botanical drug according to claim 40, wherein the mixture of plant materials is a mixture of Echinacea and Goldenseal.

43. The method for making a pharmaceutical grade botanical drug according to claim 40, wherein the mixture of plant materials is a mixture of St. John's Wort and Valerian.

44. The method for making a pharmaceutical grade botanical drug according to claim 1, 2, 3 or 4 wherein the active component is selected from the group consisting of acetogenins, alkaloids, carbohydrates, carotenoids, cinnamic acid derivatives, fatty acids, fatty acid esters, flavonoids, glycosides, isoprenoids, macrocyclic antibiotics, nucleic acids, penicillins, peptides, phenolics, polyacetylenes, polyketides, polyphenols, polysaccharides, proteins, prostaglandins, steroids and terpenoids.

45. The method for making a pharmaceutical grade botanical drug according to claim 44, wherein the active component is an acetogenin.

46. The method for making a pharmaceutical grade botanical drug according to claim 44, wherein the active component is an alkaloid.

47. The method for making a pharmaceutical grade botanical drug according to claim 44, wherein the active component is a carbohydrate.

48. The method for making a pharmaceutical grade botanical drug according to claim 44, wherein the active component is a fatty acid ester.

49. The method for making a pharmaceutical grade botanical drug according to claim 44, wherein the active component is a flavonoid.

50. The method for making a pharmaceutical grade botanical drug according to claim 44, wherein the active component is a lectin.

51. The method for making a pharmaceutical grade botanical drug according to claim 44, wherein the active component is a phenolic.

52. The method for making a pharmaceutical grade botanical drug according to claim 44, wherein the active component is a steroid.

53. The method for making a pharmaceutical grade botanical drug according to claim 44, wherein the active component is a terpinoid.

54. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the bioactivity is indicative of use for treating or ameliorating an allergic/inflammatory disorder.

55. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the bioactivity is indicative of use for treating or ameliorating with a cardiovascular disorder.

56. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the bioactivity

is indicative of use for treating or ameliorating with a central nervous system disorder.

57. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the bioactivity is indicative of use for treating or ameliorating a gastrointestinal disorder.

58. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the bioactivity is indicative of use for treating or ameliorating a metabolic disorder.

59. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the bioactivity is indicative of use for treating or ameliorating a disease induced by a microbial organism or a virus.

60. The method for making a pharmaceutical grade botanical drug according to claim 1, wherein the botanical material is a homogeneous material.

61. A method for making a pharmaceutical grade botanical drug, the method comprising the steps of:

providing a botanical material which comprises a plurality of components which have a given biological activity and wherein each component has a standardized bioactivity profile;

separating a representative aliquot from the botanical material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one of the active components;

measuring the amount of each of the active component(s) present in each of the marker fractions;

calculating the bioactivity of each of the marker fractions based on the amount of each of the active components present and the standardized component bioactivity profile to provide a calculated bioactivity fingerprint of the representative aliquot; and

comparing the calculated bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade botanical drug to determine whether the botanical material is a pharmaceutical grade botanical drug.

62. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the method comprises the additional steps of:

determining a total bioactivity of the representative aliquot of the botanical material and

comparing the total bioactivity of the representative aliquot with that of a total bioactivity of the standard to determine whether the botanical material is a pharmaceutical grade botanical drug.

63. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the botanical material is an extract made from plant material.

64. The method for making a pharmaceutical grade botanical drug according to claim 63, wherein the botanical material is an aqueous or organic extract.

65. The method for making a pharmaceutical grade botanical drug according to claim 63, wherein the botanical material is a powdered plant material.

66. The method for making a pharmaceutical grade botanical drug according to claim 61 or 62, wherein the botanical material is selected from the group consisting of Aloe, Bilberry, Black Cohosh, Chamomile, Chaste tree, Chestnut, Echinacea, Evening Primrose, Feverfew, Garlic, Ginger, Ginkgo, Ginseng (Asian), Goldenseal, Green tea, Guggulipid, Hawthorn, Ivy, Kava, Licorice, Milk Thistle, Passion Flower, Pumpkin, Pygeum, Rosemary, Siberian Ginseng, Saw Palmetto, St. John's Wort, Stinging Nettle and Valerian.

67. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the botanical material is a mixture of plant materials.

68. The method for making a pharmaceutical grade botanical drug according to claim 61 or 62, wherein the active component is selected from the group consisting of acetogenins, alkaloids, carbohydrates, carotenoids, cinnamic acid derivatives, fatty acids, fatty acid esters, flavonoid, glycosides, isoprenoids, macrocyclic antibiotics, nucleic acids, penicillins, peptides, phenolics, polyacetylenes, polyketides, polyphenols, polysaccharides, proteins, prostaglandins, steroids and terpenoids.

69. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the bioactivity is indicative of use for treating or ameliorating an allergic/inflammatory disorder.

70. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the bioactivity is indicative of use for treating or ameliorating a cardiovascular disorder.

71. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the bioactivity is indicative of use for treating or ameliorating a central nervous system disorder.

72. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the bioactivity is indicative of use for treating or ameliorating a gastrointestinal disorder.

73. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the bioactivity is indicative of use for treating or ameliorating a metabolic disorder.

74. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the bioactivity is indicative of use for treating or ameliorating a disease induced by a microbial organism or a virus.

75. The method for making a pharmaceutical grade botanical drug according to claim 61, wherein the botanical material is a homogeneous material.

76. The method for making a pharmaceutical grade botanical drug according to claim 1, 4, 61 or 62, wherein the marker fractions comprise a class of related components.

77. A method for making a pharmaceutical grade botanical drug, the method comprising the steps of:

providing a botanical material which has a given biological activity, said botanical material comprising a plurality of components;

separating a representative aliquot of the botanical material into a plurality of marker fractions wherein at least one of the marker fractions comprises at least one active class of components;

determining the degree of the given biological activity for each of the marker fractions to provide a bioactivity fingerprint of the representative aliquot; and

comparing the bioactivity fingerprint of the representative aliquot to a bioactivity fingerprint standard which has been established for a pharmaceutical grade botanical drug to determine whether the botanical material is a pharmaceutical grade botanical drug.

78. The method for making a pharmaceutical grade botanical drug according to claim 77, wherein the botanical material is selected from the group consisting of Aloe, Bilberry, Black Cohosh, Chamomile, Chaste tree, Chestnut, Echinacea, Evening Primrose, Feverfew, Garlic, Ginger, Ginkgo, Ginseng (Asian), Goldenseal, Green tea, Guggulipid, Hawthorn, Ivy, Kava, Licorice, Milk Thistle, Passion Flower, Pumpkin, Pygeum, Rosemary, Siberian Ginseng, Saw Palmetto, St. John's Wort, Stinging Nettle and Valerian.

79. The method for making a pharmaceutical grade botanical drug according to claim 77, wherein the active class of components are selected from the group consisting of acetogenins, alkaloids, carbohydrates, carotenoids, cinnamic acid derivatives, fatty acids, fatty acid esters, flavonoids, glycosides, isoprenoids, macrocyclic antibiotics, nucleic acids, penicillins, peptides, phenolics, polyacetylenes, polyketides, polyphenols, polysaccharides, proteins, prostaglandins, steroids and terpenoids.

80. A pharmaceutical grade botanical drug made by the method according to claims 1, 3, 61 or 77.

81. A pharmaceutical grade botanical drug made by the method according to claim 1, wherein the marker fractions comprise a class of related components.

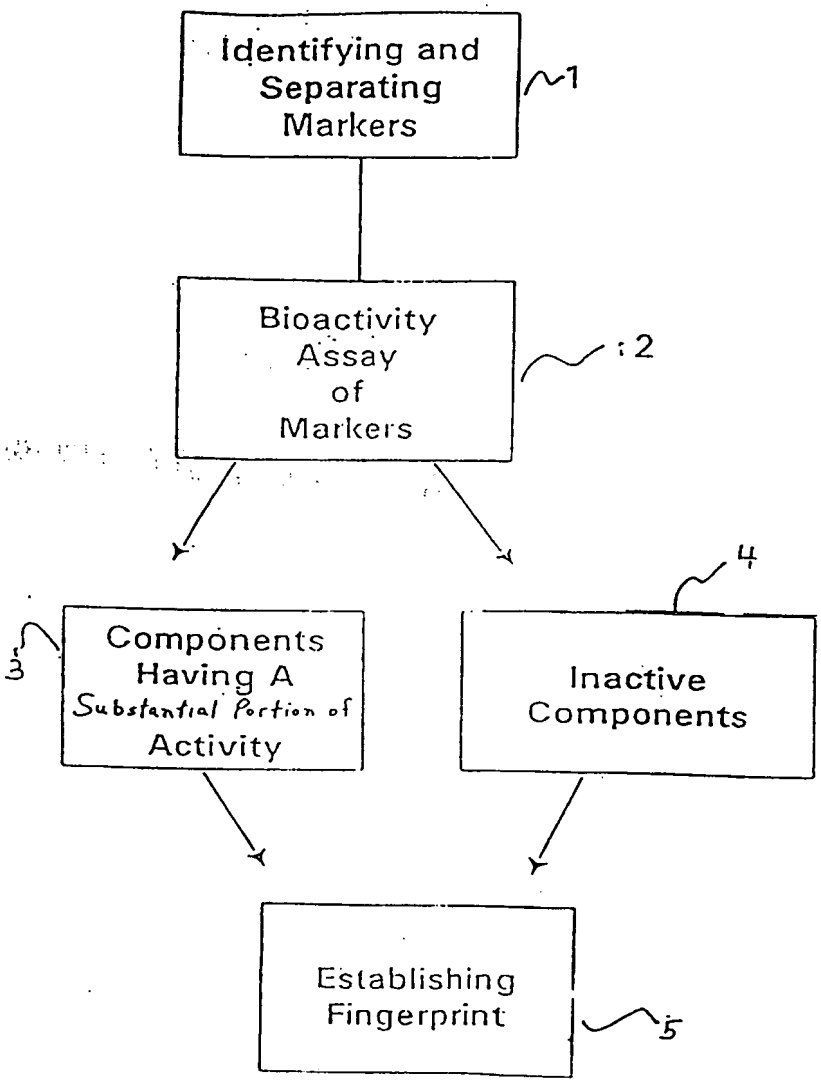

FIG. 1

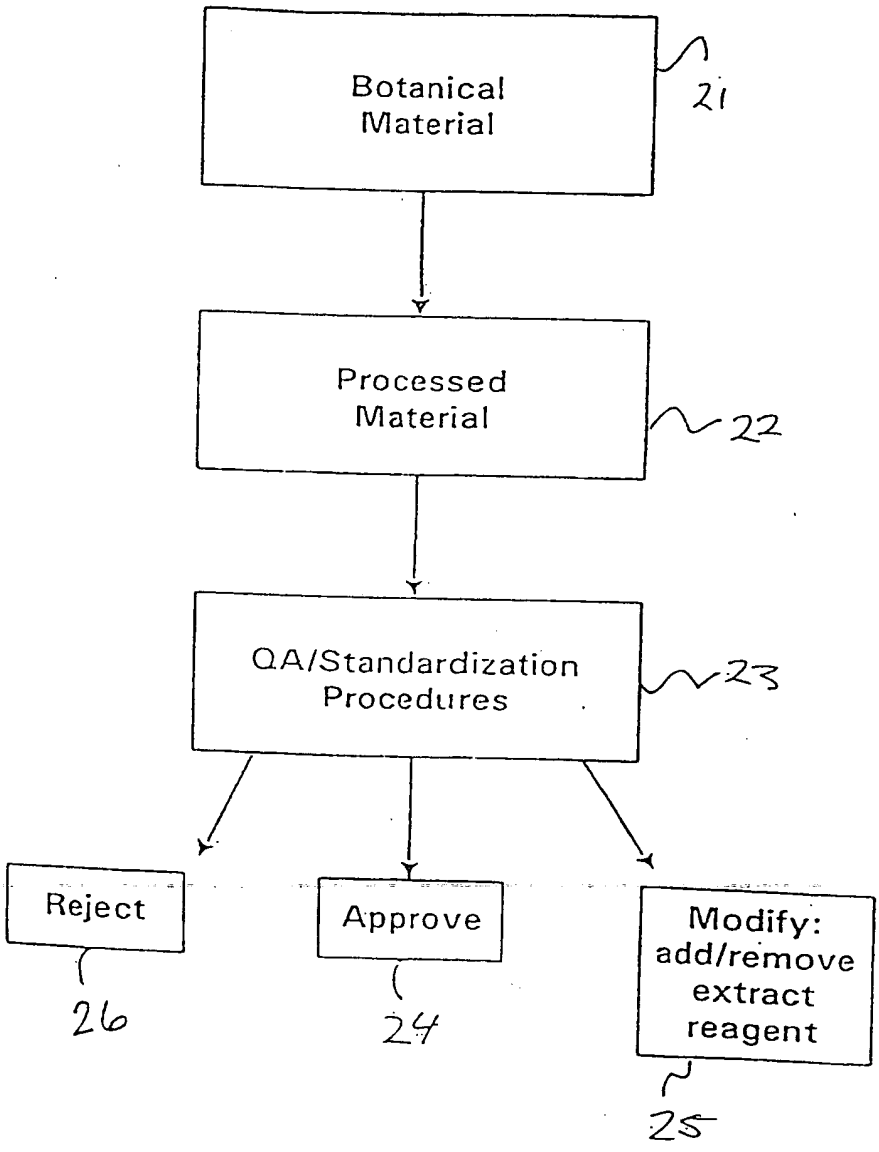

FIG. 2

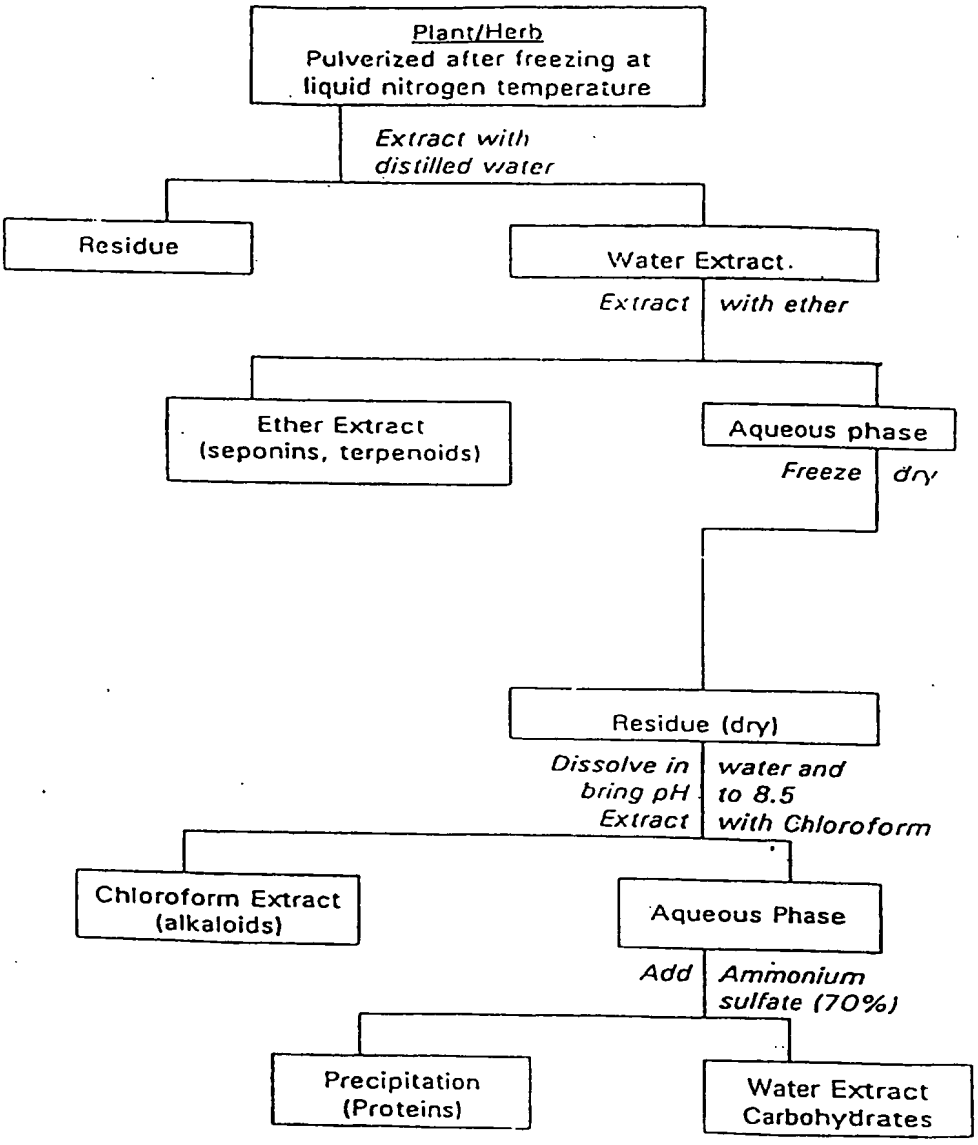

FIG. 3

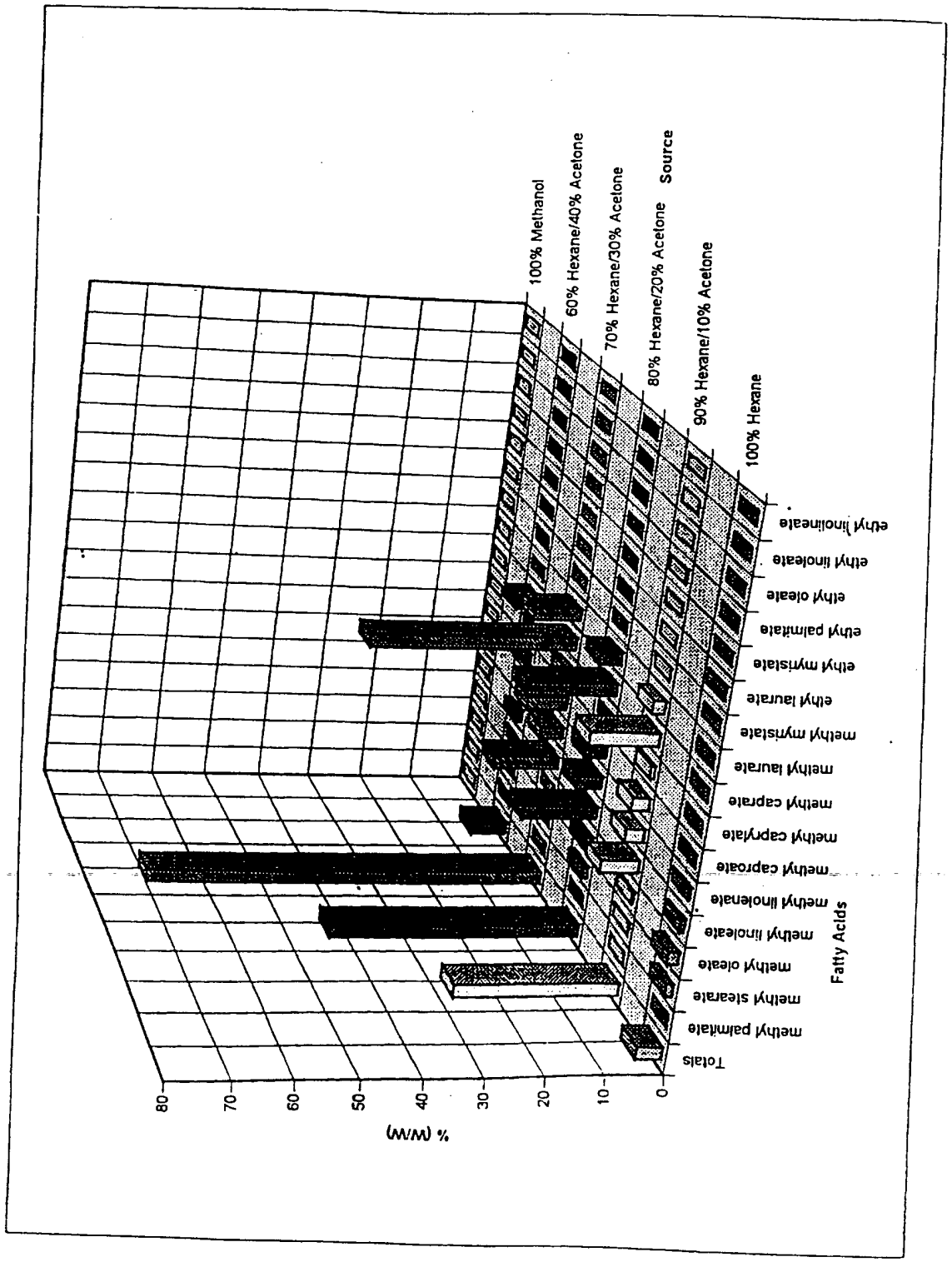

FIG. 4

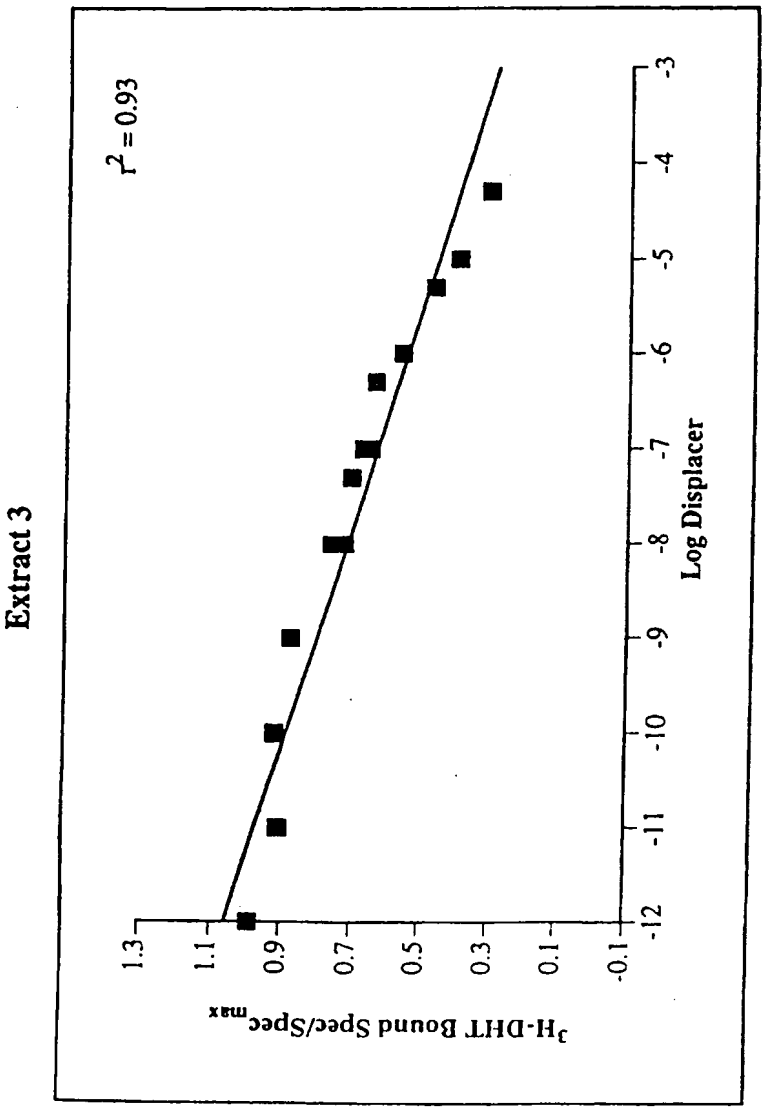

FIG. 5

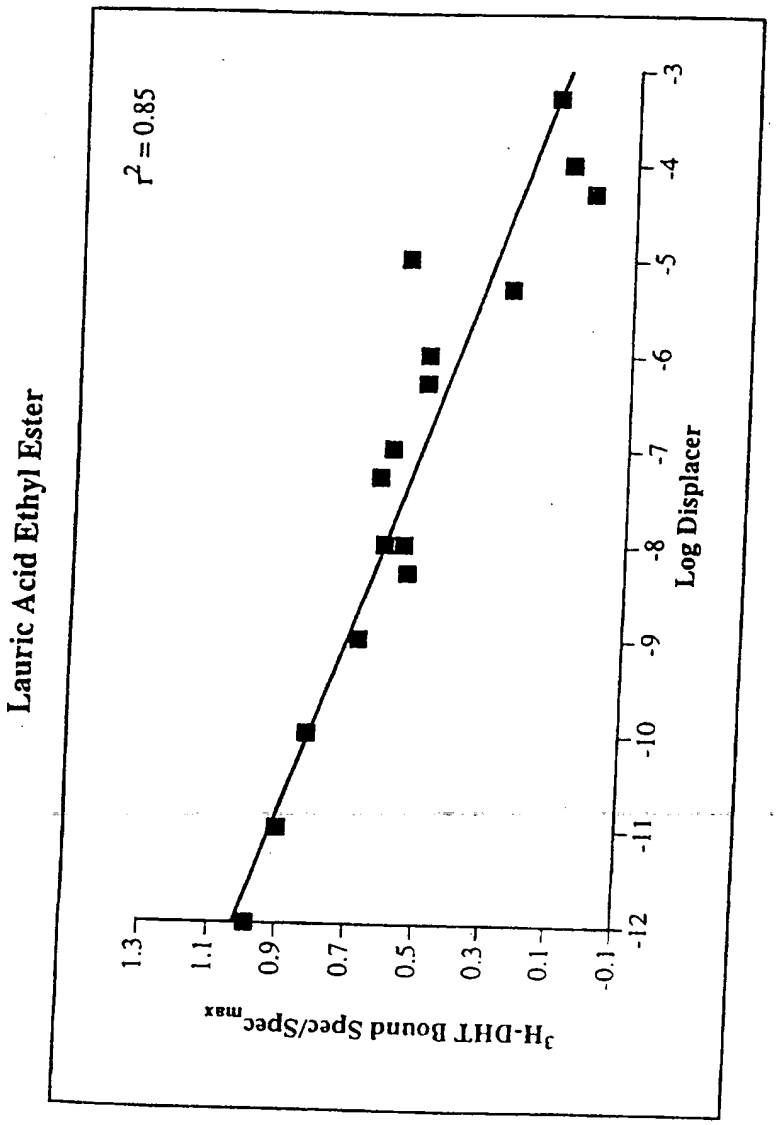

FIG. 6

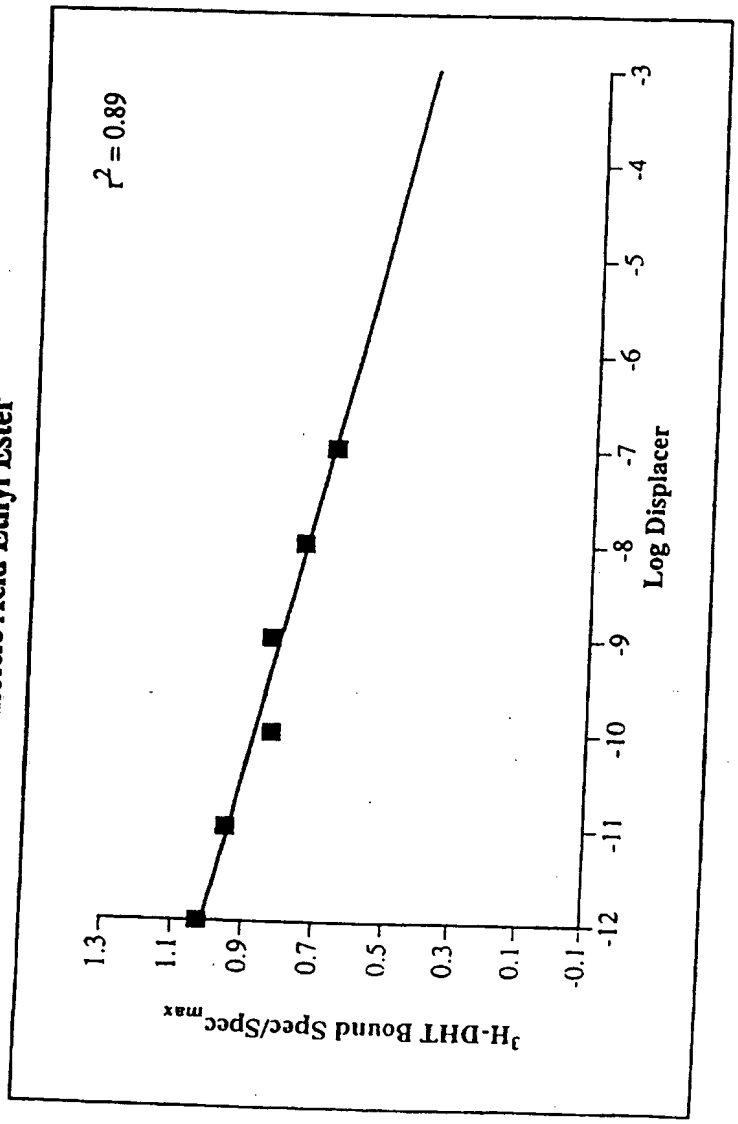

FIG. 7

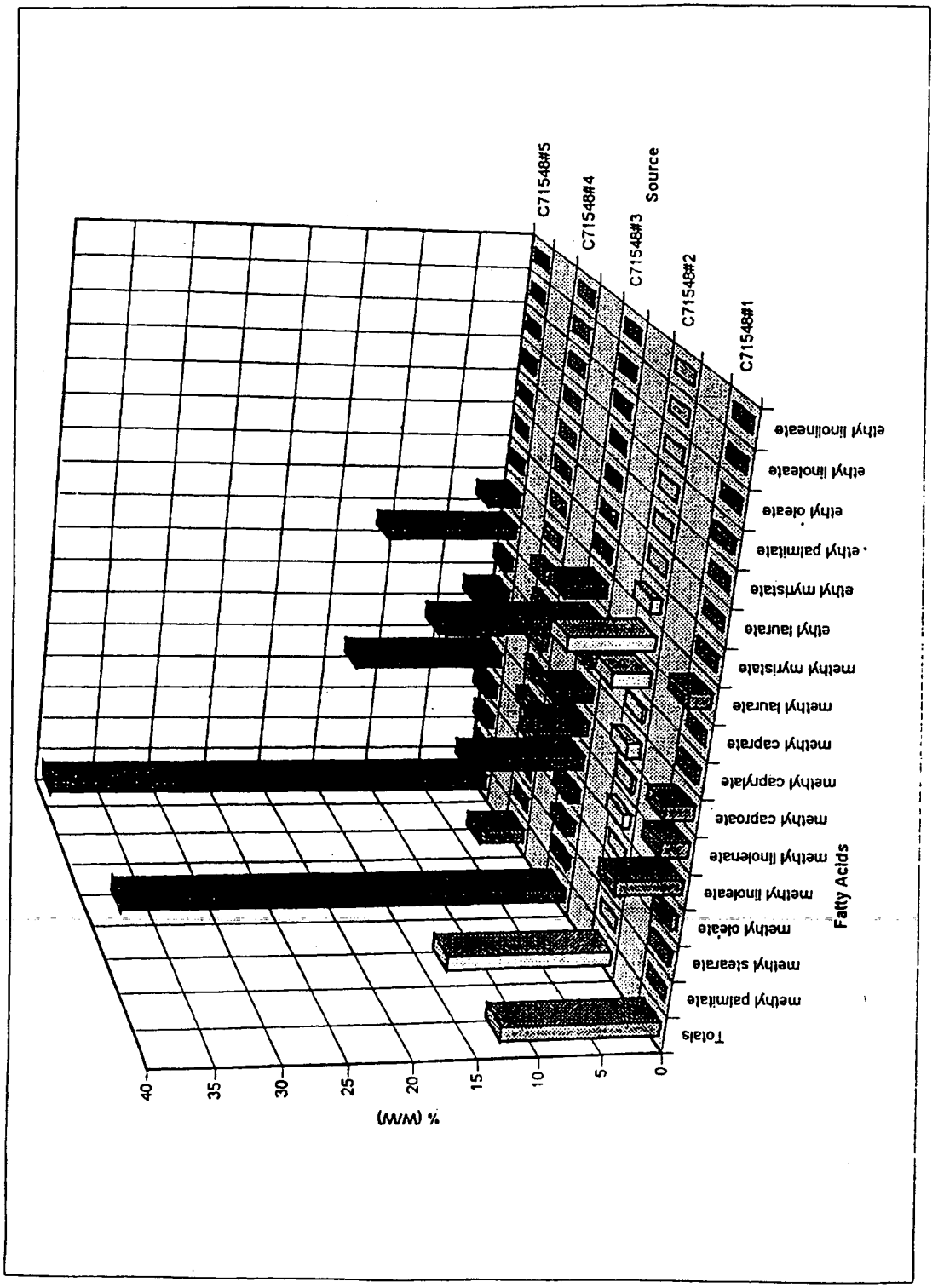

FIG. 8

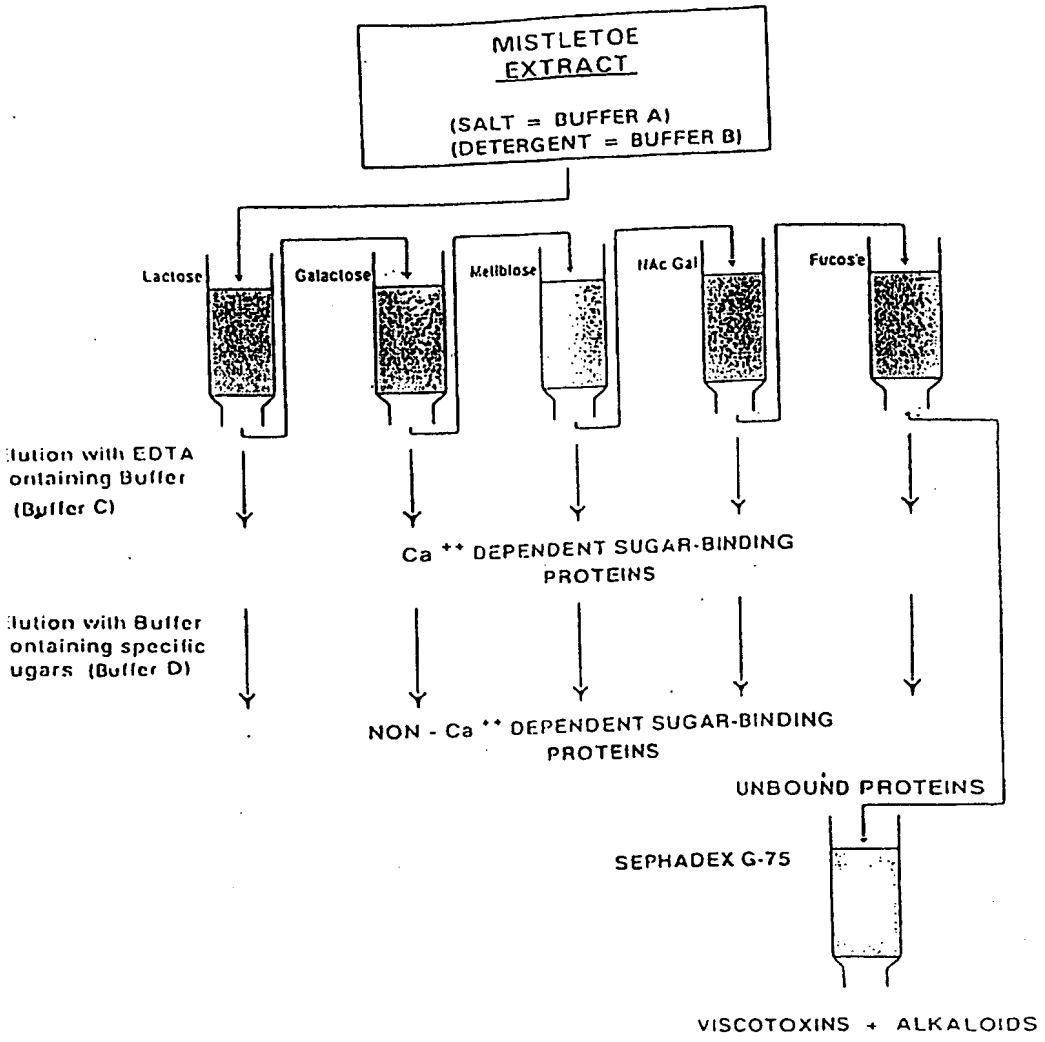

FIG. 9

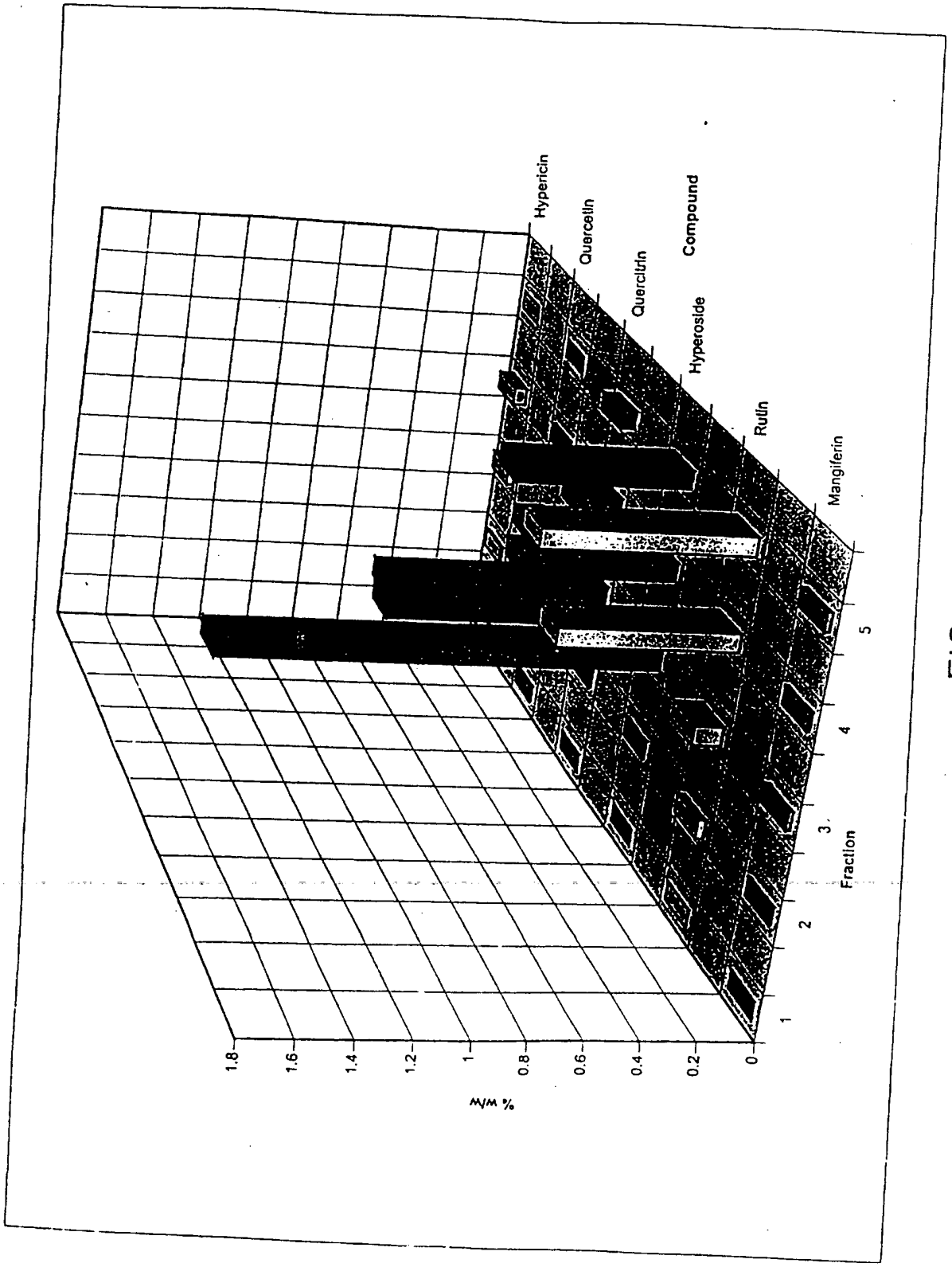

FIG. 10

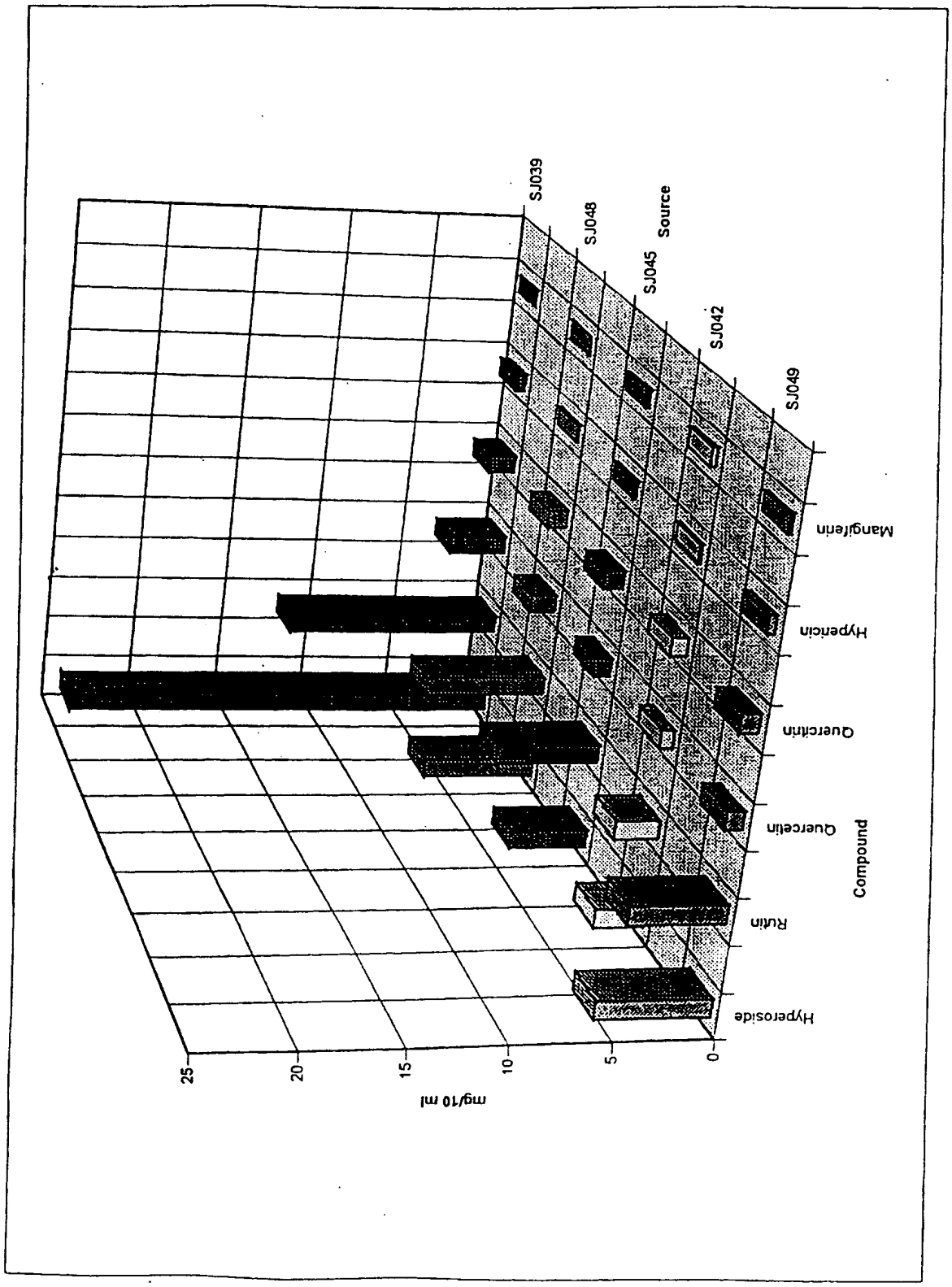

FIG. 11

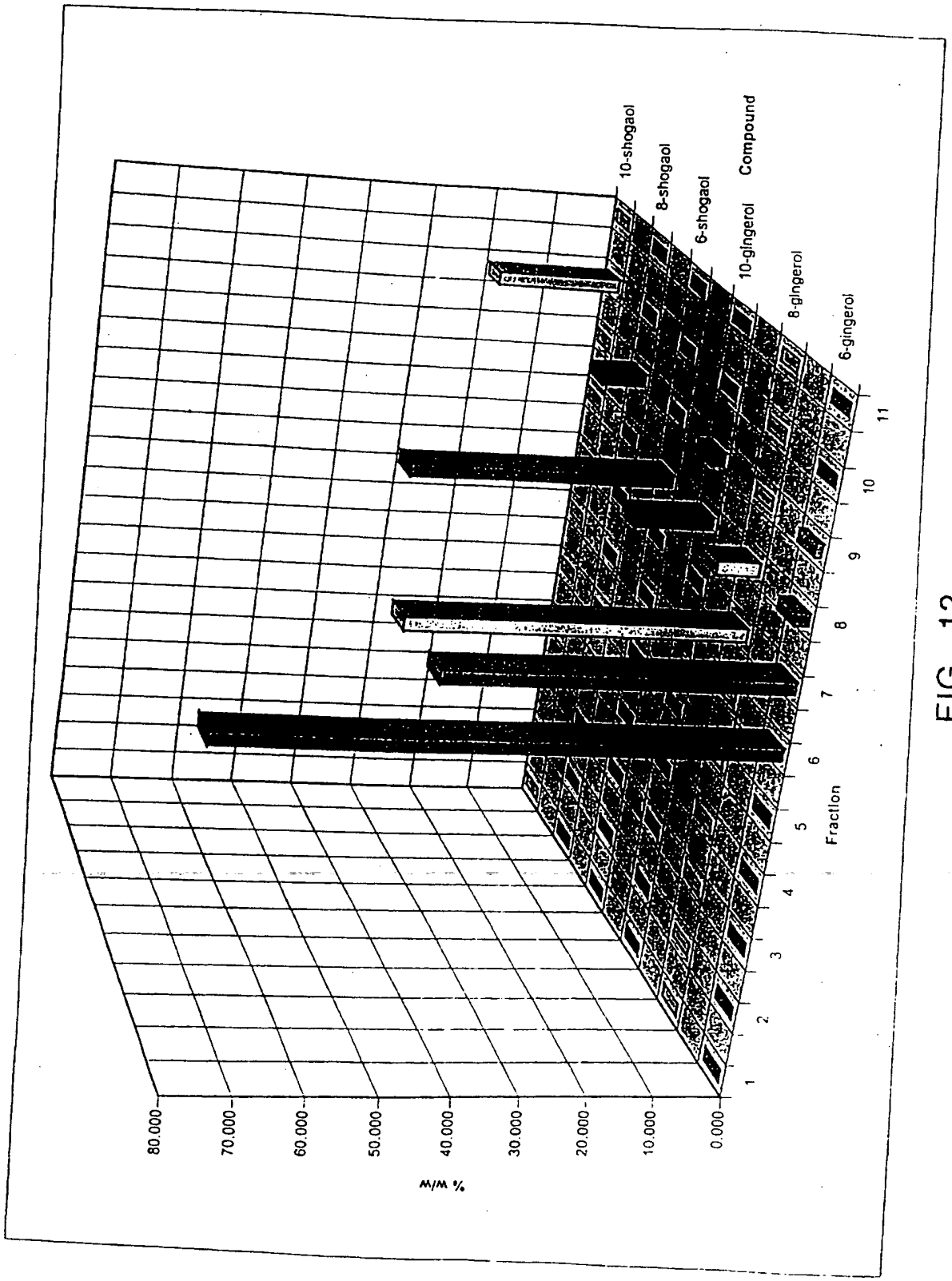

FIG. 12

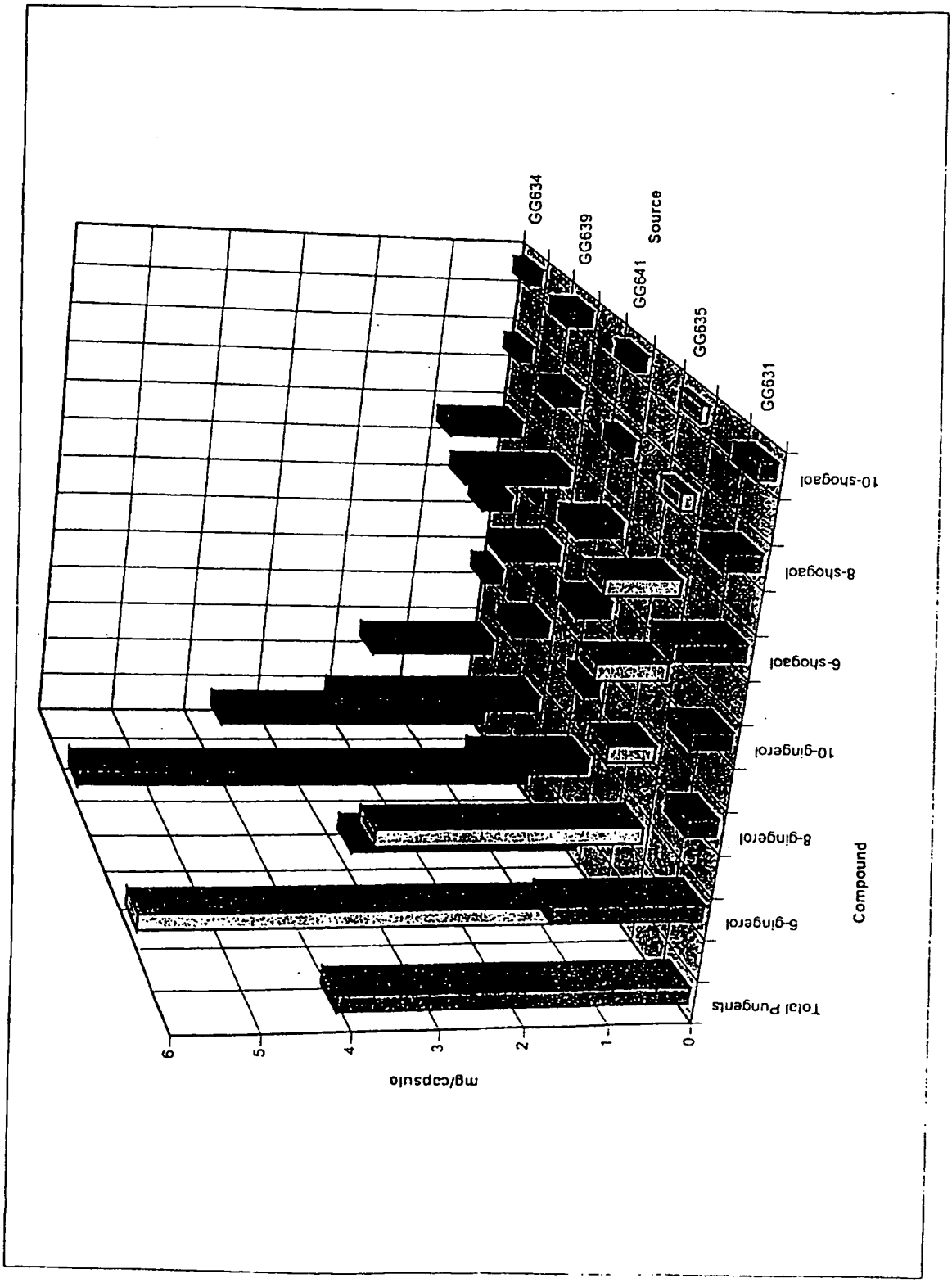

FIG. 13

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 9,/06988

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 A61K35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 32122 A (UNIVERSITY OF SOUTHERN CALIFORNIA) 17 October 1996 cited in the application see the whole document	1-81
P,X	US 5 547 674 A (T. A. KHWAJA) 20 August 1996 see the whole document	1-81
A	GB 1 152 618 A (CIBA LIMITED) 21 May 1969 see the whole document	1-81
A	US 3 394 120 A (F. VESTER) 23 July 1968 see the whole document	1-81
A	US 3 472 831 A (F. VESTER ET AL.) 14 October 1969 see the whole document	1-81

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
19 August 1997	27 -08- 1997
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Griffith, G

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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